THE
BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

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AUGUST, 1956

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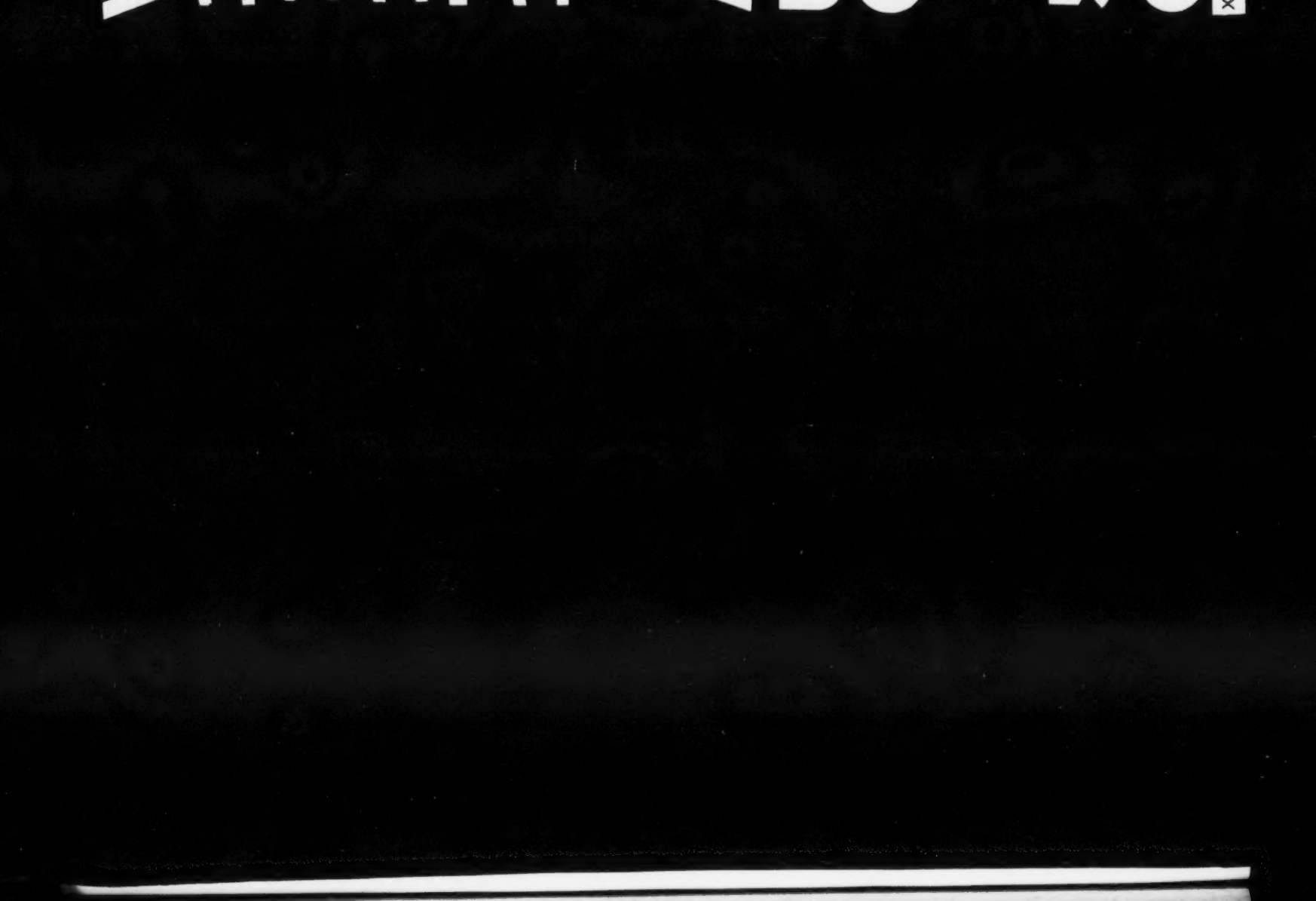

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THE BIOLOGICAL BULLETIN

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THE MARINE BIOLOGICAL LABORATORY

FIFTY-EIGHTH REPORT, FOR THE YEAR 1955—SIXTY-EIGHTH YEAR

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II. ACT OF INCORPORATION

No. 3170

Commonwealth of Massachusetts

Be It Known, That whereas Alpheus Hyatt, William Sanford Stevens, William T. Sedgwick, Edward G. Gardiner, Susan Minns, Charles Sedgwick Minot, Samuel Wells, William G. Farlow, Anna D. Phillips, and B. H. Van Vleck have associated themselves with the intention of forming a Corporation under the name of the Marine Biological Laboratory, for the purpose of establishing and maintaining a laboratory or station for

scientific study and investigation, and a school for instruction in biology and natural history, and have complied with the provisions of the statutes of this Commonwealth in such case made and provided, as appears from the certificate of the President, Treasurer, and Trustees of said Corporation, duly approved by the Commissioner of Corporations, and recorded in this office;

Now, therefore, I, HENRY B. PIERCE, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardiner, S. Minns, C. S. Minot, S. Wells, W. G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and successors, are legally organized and established as, and are hereby made, an existing Corporation, under the name of the MARINE BIOLOGICAL LABORATORY, with the powers, rights, and privileges, and subject to the limitations, duties, and restrictions, which by law appertain thereto.

Witness my official signature hereunto subscribed, and the seal of the Commonwealth of Massachusetts hereunto affixed, this twentieth day of March, in the year of our Lord One Thousand Eight Hundred and Eighty-Eight.

[SEAL]

HENRY B. PIERCE,
Secretary of the Commonwealth.

III. BY-LAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

I. The members of the Corporation shall consist of persons elected by the Board of Trustees.

II. The officers of the Corporation shall consist of a President, Vice President, Director, Treasurer, and Clerk.

III. The Annual Meeting of the members shall be held on the Friday following the second Tuesday in August in each year at the Laboratory in Woods Hole, Massachusetts, at 9:30 A.M., and at such meeting the members shall choose by ballot a Treasurer and a Clerk to serve one year, and eight Trustees to serve four years, and shall transact such other business as may properly come before the meeting. Special meetings of the members may be called by the Trustees to be held at such time and place as may be designated.

IV. Twenty-five members shall constitute a quorum at any meeting.

V. Any member in good standing may vote at any meeting, either in person or by proxy duly executed.

VI. Inasmuch as the time and place of the Annual Meeting of members are fixed by these By-laws, no notice of the Annual Meeting need be given. Notice of any special meeting of members, however, shall be given by the Clerk by mailing notice of the time and place and purpose of such meeting, at least fifteen (15) days before such meeting, to each member at his or her address as shown on the records of the Corporation.

VII. The Annual Meeting of the Trustees shall be held promptly after the Annual Meeting of the Corporation at the Laboratory in Woods Hole, Mass. Special meetings of the Trustees shall be called by the President, or by any seven Trustees, to be held at such time and place as may be designated, and the Secretary shall give notice thereof by written or printed notice, mailed to each Trustee at his address as shown on the records of the Corporation, at least one (1) week before the meeting. At such special meeting

only matters stated in the notice shall be considered. Seven Trustees of those eligible to vote shall constitute a quorum for the transaction of business at any meeting.

VIII. There shall be three groups of Trustees:

(A) Thirty-two Trustees chosen by the Corporation, divided into four classes, each to serve four years. After having served two consecutive terms of four years each, Trustees are ineligible for re-election until a year has elapsed. In addition, there shall be two groups of Trustees as follows:

(B) Trustees ex officio, who shall be the President and Vice President of the Corporation, the Director of the Laboratory, the Associate Director, the Treasurer, and the Clerk;

(C) Trustees Emeriti, who shall be elected from *present* or *former* Trustees by the Corporation. Any regular Trustee who has attained the age of seventy years shall continue to serve as Trustee until the next Annual Meeting of the Corporation, whereupon his office as regular Trustee shall become vacant and be filled by election by the Corporation and he shall become eligible for election as Trustee *Emeritus* for life. The Trustees *ex officio* and *Emeritus* shall have all the rights of the Trustees except that Trustees *Emeritus* shall not have the right to vote.

The Trustees and officers shall hold their respective offices until their successors are chosen and have qualified in their stead.

IX. The Trustees shall have the control and management of the affairs of the Corporation; they shall elect a President of the Corporation who shall also be Chairman of the Board of Trustees and who shall be elected for a term of five years and shall serve until his successor is selected and qualified; and shall also elect a Vice President of the Corporation who shall also be the Vice Chairman of the Board of Trustees and who shall be selected for a term of five years and shall serve until his successor is selected and qualified; they shall appoint a Director of the Laboratory; and they may choose such other officers and agents as they may think best; they may fix the compensation and define the duties of all the officers and agents; and may remove them, or any of them, except those chosen by the members, at any time; they may fill vacancies occurring in any manner in their own number or in any of the offices. The Board of Trustees shall have the power to choose an Executive Committee from their own number, and to delegate to such Committee such of their own powers as they may deem expedient. They shall from time to time elect members to the Corporation upon such terms and conditions as they may think best.

X. The Associates of the Marine Biological Laboratory shall be an unincorporated group of persons (including associations and corporations) interested in the Laboratory and shall be organized and operated under the general supervision and authority of the Trustees.

XI. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees.

XII. The account of the Treasurer shall be audited annually by a certified public accountant.

XIII. These By-laws may be altered at any meeting of the Trustees, provided that the notice of such meeting shall state that an alteration of the By-laws will be acted upon.

IV. REPORT OF THE DIRECTOR

To the Trustees of the Marine Biological Laboratory:

Gentlemen:

I submit herewith the report of the sixty-eighth session of the Laboratory.

Except during some of the World War II years, the Laboratory has always operated at capacity in the summer months. However, there have always been more applicants for research space than could be accommodated. The total number of investigators at the Laboratory for the past few years has not been as great as immediately after the war. This has been due in part to the diversion of some laboratories from research to special uses such as radioisotope laboratories, electron-microscopy, and for special equipment, in all, some six laboratories. Preference is given to those investigators who can devote the major share of the summer to their projects and who use the marine biological materials available in the Woods Hole area. However, there is not a rigid adherence to these two factors. The Laboratory is primarily interested in fostering and promoting outstanding biological research, in its broadest terms, and in developing scientists. The allotment of space, however, is becoming more difficult due to an increasing number of applications for research space. For this year an increased number of well qualified investigators cannot be accommodated.

1. Plant Changes and Improvements

Through a grant of $50,000 from the National Science Foundation the extensive repairs of the hurricane damage of 1954 were completed and some modifications were made in the Main Building and Pump House to prevent a recurrence of the flooding of these buildings such as occurred in the 1954 hurricanes. Included were the bricking up of some of the basement windows in the Main Building and in the Pump House as well as other stand-by measures to be used in an emergency. It was necessary to replace and relocate considerable equipment which was irreparably damaged by salt water.

2. Pension Plan

During the past year a study was made of a number of pension plans in force in other organizations with a view to developing a pension plan for the full-time employees of the Laboratory. A plan was developed and became effective September 1, 1955. The employees of the Laboratory were already under Social Security. The Laboratory's pension plan will supplement Social Security benefits. The entire cost of the pension plan will be borne by the Laboratory. For the past 26 years the Laboratory has put aside annually amounts up to 10% of the payroll for pension purposes so there have accumulated funds which are adequate to carry the plan.

3. Grants, Contracts and Contributions

The total income from these sources of support amounted to $192,555.94 in 1955. This represents 39% of the total Laboratory budget and consists of the following accounts:

Amer. Cancer Soc.—026—Function of Nuclei and Nucleic Acids	$ 13,500.00
Amer. Cancer Soc.—R–7F—Fundamental Studies in Radiobiology ..	6,600.00
A. E. C.–M. B. L.—At 30–1–1343—Program of Research on the Physiology of Marine Organisms Using Radioisotopes	7,674.00
N. I. H.—B6430—Encephalization in Embryonic Development	1,998.00
N. I. H.—SA43PH 423—Investigations of the Microscopic Physiology of Various Forms of Living Marine Life	1,350.00
N. I. H.—B799—Electrical and Mechanical Changes in Muscle	864.00
N. I. H.—RG4359—Biological Research on the Morphology, Ecology, Physiology, Biochemistry and Biophysics of Marine Organisms ..	40,000.00
National Sci. Found.—G1469—Provision of Funds for Scientific Equipment and Facilities for Biological Research	50,000.00
National Sci. Found.—G1807—Mechano-Chemical Coupling in Muscle	11,500.00
National Sci. Found.—G1395—Osmoregulation of Excretion in Tunicates	1,708.04
O. N. R.—RG4359—Studies in Marine Biology	15,000.00
O. N. R.—09701—Studies on Isolated Nerve Fibers	6,359.34
O. N. R.—09702—Investigation of Environmental Factors Influencing Certain Marine Biological Populations in the Woods Hole Area	4,437.56
American Philosophical Soc.	2,500.00
M. B. L. Associates	2,940.00
Eli Lilly Co.	5,000.00
Rockefeller Found.	20,000.00
Other	1,125.00
	$192,555.94

4. Instruction

Dr. Daniel Mazia completed five years as head of the course in Physiology in 1955 and will be succeeded by Dr. Stephen Kuffler. Dr. S. Meryl Rose also completed a five-year term as head of the course in Embryology and will be succeeded by Dr. Mac V. Edds. The success of these courses is attested by the large number of applicants for admission to both of the courses. The Laboratory is to be congratulated in having such excellent leadership in its various courses.

5. Fellowships and Scholarships

Under a new plan all of the Lalor Fellows will be selected by a panel set up by the Lalor Foundation. The successful applicants who elect to work at the Marine Biological Laboratory will be permitted to do so. It is hoped that under this new plan the Laboratory will continue to have a fair number of Lalor Fellows. The Laboratory has much to offer these young postdoctoral fellows and would not realize its full educational potentialities without them. Also these fellowships have been of real value to the Laboratory, resulting in the recruitment of highly qualified investigators in subsequent years.

6. Losses by Death

Since the last report the Laboratory has lost through death two eminent scientists who served for many years on the Board of Trustees, Professor George Howard Parker and Professor Warder Clyde Allee. Both of these scientists contributed in an effective way to the prestige and development of the Laboratory.

Respectfully submitted,

PHILIP B. ARMSTRONG,
Director

1. THE STAFF, 1955

PHILIP B. ARMSTRONG, Director, State University of New York, School of Medicine, Syracuse

SENIOR STAFF OF INVESTIGATION

A. P. MATHEWS, Professor of Biochemistry, *Emeritus*, University of Cincinnati
G. H. PARKER, Professor of Zoology, *Emeritus*, Harvard University

ZOOLOGY

I. CONSULTANTS

F. A. BROWN, JR., Professor of Zoology, Northwestern University
LIBBIE H. HYMAN, American Museum of Natural History
A. C. REDFIELD, Woods Hole Oceanographic Institution

II. INSTRUCTORS

THEODORE H. BULLOCK, Associate Professor of Zoology, University of California, Los Angeles, in charge of course
JOHN H. LOCHHEAD, Associate Professor of Zoology, University of Vermont
NORMAN A. MEINKOTH, Associate Professor of Zoology, Swarthmore College
GROVER STEPHENS, Assistant Professor of Zoology, University of Minnesota
JOHN M. ANDERSON, Associate Professor of Zoology, Cornell University
MURIEL SANDEEN, Department of Zoology, Duke University
L. M. PASSANO, Department of Zoology, University of Washington, Seattle
MORRIS ROCKSTEIN, Department of Physiology, New York University, Bellevue Medical Center

III. LABORATORY ASSISTANTS

ALLISON BURNETT, Ithaca, New York
DOROTHY M. SKINNER, Tufts College

EMBRYOLOGY

I. INSTRUCTORS

S. MERYL ROSE, Associate Professor of Zoology, University of Illinois, in charge of course
CLIFFORD GROBSTEIN, National Cancer Institute
MAC V. EDDS, JR., Associate Professor of Biology, Brown University
NELSON T. SPRATT, JR., Professor of Zoology, University of Minnesota
J. P. TRINKAUS, Assistant Professor of Zoology, Yale University
EDGAR ZWILLING, Associate Professor of Genetics, University of Connecticut

II. Laboratory Assistants

Joan K. Ericksen, Radcliffe College
Roger D. Milkman, Harvard University

PHYSIOLOGY

I. Consultants

Merkel H. Jacobs, Professor of Physiology, University of Pennsylvania
Otto Loewi, Professor of Pharmacology, New York University, School of Medicine
Arthur K. Parpart, Professor of Biology, Princeton University
Albert Szent-Gyorgyi, Director, Institute for Muscle Research, Woods Hole
E. S. Guzman Barron, Associate Professor of Biochemistry, University of Chicago

II. Instructors

Daniel Mazia, Associate Professor of Zoology, University of California, in charge of course
Herman M. Kalckar, National Institutes of Health
Stephen Kuffler, Associate Professor of Ophthalmology, Wilmer Institute, Johns Hopkins University Medical School
Max A. Lauffer, Professor and Head of Dept. of Biophysics, University of Pittsburgh
George Wald, Professor of Biology, Harvard University
Andrew Szent-Gyorgyi, Independent Investigator, The Institute for Muscle Research

III. Laboratory Assistant

Paul Bernstein, Department of Zoology, Columbia University

BOTANY

I. Consultants

Maxwell S. Doty, Associate Professor of Botany, University of Hawaii
Wm. Randolph Taylor, Professor of Botany, University of Michigan

II. Instructors

Harold C. Bold, Professor of Biology, Vanderbilt University, in charge of course
Robert W. Krauss, University of Maryland
Richard C. Starr, Instructor in Botany, University of Indiana

III. Laboratory Assistants

Francis R. Trainor, Vanderbilt University

IV. Collector

Ann Allen, University of Indiana

MARINE ECOLOGY

I. Consultants

Alfred C. Redfield, Woods Hole Oceanographic Institution

II. Instructors

Bostwick H. Ketchum, Marine Microbiologist, Woods Hole Oceanographic Institution, in charge of course
Edwin T. Moul, Assistant Professor of Botany, Rutgers University
Charles Jenner, Associate Professor of Zoology, University of North Carolina

III. Assistant

Rudolf Scheltema, George Washington University

THE LABORATORY STAFF, 1955

Homer P. Smith, General Manager

Mrs. Deborah Lawrence Harlow, Librarian
James McInnis, Manager of Supply Department
Robert Kahler, Superintendent, Buildings and Grounds
Robert B. Mills, Manager, Department of Research Service

GENERAL OFFICE

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Mrs. Lila Myers
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LIBRARY

Mary E. Castellano, Assistant Librarian
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DEPARTMENT OF RESEARCH SERVICE

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John P. Harlow
Seaver Harlow
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Ruth S. Crowell
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Robert Perry
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Robert O. Lehy
Carl Schweidenback
Bruno Trapasso
James P. Whitcomb
H. S. Wagstaff

BIOLOGICAL BULLETIN

DONALD P. COSTELLO, Managing Editor
University of North Carolina, Dept. of Zoology
Chapel Hill, North Carolina
CATHERINE HENLEY, Assistant to the Editor

2. INVESTIGATORS AND STUDENTS

Independent Investigators, 1955

ABBOTT, ROBINSON SHEWELL, Assistant Professor of Botany, Cornell University
AGNEW, L. R. C., Research Fellow, Department of Nutrition, Harvard University
ALLEN, ROBERT DAY, Instructor in Zoology, University of Michigan
ANDERSON, JOHN MAXWELL, Associate Professor of Zoology, Cornell University
ARMSTRONG, PHILIP B., Professor of Anatomy, State Univ. of New York, College of Medicine
ARNOLD, WILLIAM A., Scientific Investigator, Oak Ridge National Laboratory
BARTON, JAY, Assistant Professor of Zoology, Columbia University
BENNETT, MIRIAM F., Instructor in Biology, Sweet Briar College
BERGER, CHARLES A., Chairman, Department of Biology, Fordham University
BLUM, HAROLD E., Physiologist, Princeton University
BOETTIGER, EDWARD G., Associate Professor, University of Connecticut
BOLD, HAROLD C., Vanderbilt University
BRIDGMAN, JOSEPHINE, Professor of Biology, Agnes Scott College
BROWN, FRANK A., JR., Chairman, Dept. of Biological Sciences, Northwestern University
BRUMMETT, ANNA RUTH, Instructor in Biology, Carleton College
BULLOCK, THEODORE H., Associate Professor of Zoology, Univ. of California, Los Angeles
BURBANCK, W. D., Department of Biology, Emory University
BUTLER, ELMER G., Professor of Zoology, Princeton University
CHAET, A. B., Instructor in Zoology, University of Maine
CHANG, C. Y., Research Associate, State University of Iowa
CHASE, AURIN M., Associate Professor of Biology, Princeton University
CHENEY, RALPH HOLT, Professor of Biology, Brooklyn College
CLAFF, C. LLOYD, Research Associate in Surgery, Harvard Medical School
CLARK, ELLIOT R., Professor *Emeritus* of Anatomy, Univ. of Pennsylvania School of Medicine
CLAYTON, RODERICK K., Associate Professor of Physics, U. S. Naval P. G. School
CLOWES, G. H. A., Research Director *Emeritus*, Eli Lilly and Company
COLE, KENNETH S., Chief, Laboratory of Biophysics, National Institutes of Health
COLWIN, ARTHUR L., Associate Professor and Lecturer, Queens College
COOPERSTEIN, SHERWIN J., Assistant Professor of Anatomy, Western Reserve Univ. School of Medicine
CORLISS, CLARK E., Instructor in Anatomy, University of Tennessee
COSTELLO, DONALD P., Kenan Professor of Zoology, Univ. of North Carolina
CROWELL, SEARS, Assistant Professor of Zoology, Indiana University
CSAPO, A., Carnegie Institution of Washington
DWYER, JOHN D., Director, Dept. of Biology, St. Louis University
EDDS, M. V., JR., Associate Professor of Biology, Brown University
ELLIOTT, ALFRED M., Associate Professor of Zoology, University of Michigan
FAILLA, G., Professor, Columbia University
FITZHUGH, RICHARD, Instructor in Physiological Optics, Johns Hopkins University
FLAVIN, MARTIN, JR., Postdoctoral Fellow, New York University
FREYGANG, WALTER H., JR., S. A. Surg. (R) U. S. Public Health Service
GALL, JOSEPH G., Instructor in Zoology, University of Minnesota
GAMOW, GEORGE, Professor, George Washington University
GASTEIGER, EDGAR L., Assistant Professor of Physiology, Harvard Medical School
GILMAN, LAUREN C., Associate Professor of Zoology, University of Miami
GREEN, JAMES W., Assistant Professor of Physiology, Rutgers University

GREEN, MAURICE, Instructor of Biochemistry, Childrens Hospital, University of Pennsylvania
GREGG, JAMES H., Assistant Professor of Biology, University of Florida
GROBSTEIN, CLIFFORD, Biologist, National Cancer Institute
GROSCH, DANIEL S., Associate Professor of Genetics, North Carolina State College
GRUNDFEST, HARRY, Associate Professor of Neurology, College of Physicians and Surgeons
GUTTMAN, RITA, Assistant Professor of Biology, Brooklyn College
HAGIWARA, SUSUMU, Research Associate, University of California, Los Angeles
HARVEY, ETHEL BROWNE, Independent Investigator, Biology Department, Princeton University
HARVEY, E. NEWTON, Professor of Physiology, Princeton, New Jersey
HAY, ELIZABETH D., Instructor in Anatomy, Johns Hopkins University School of Medicine
HAYWOOD, CHARLOTTE, Professor of Physiology, Mount Holyoke College
HEILBRUNN, L. V., Zoological Laboratory, University of Pennsylvania
HERVEY, JOHN P., Electronic Engineer, Rockefeller Institute for Medical Research
HOLZ, GEORGE G., JR., Assistant Professor of Zoology, Syracuse University
HOWARD, ROBERT STEARNS, Assistant Professor of Biology, University of Delaware
HYDE, BEAL B., Assistant Professor of Plant Sciences, University of Oklahoma
JACOBS, M. H., Professor of General Physiology, Medical School, University of Pennsylvania
JENNER, CHARLES E., Associate Professor of Zoology, University of North Carolina
JENKINS, GEORGE B., *Emeritus* Professor of Anatomy, George Washington University
KALCKAR, HERMAN M., Visiting Scientist, National Institutes of Health
KAVANAU, J. LEE, Assistant, Rockefeller Institute for Medical Research
KEMPTON, RUDOLF T., Professor of Zoology, Vassar College
KEOSIAN, JOHN, Professor of Biology, Rutgers University
KIND, C. ALBERT, Assistant Professor of Zoology, University of Connecticut
KING, JOHN W., Professor of Biology, Morgan State College
KLEINHOLZ, L. H., Professor of Biology, Reed College
KLOTZ, IRVING M., Professor of Chemistry, Northwestern University
KRAHL, MAURICE E., Professor of Physiology, University of Chicago
KUFFLER, STEPHEN W., Associate Professor of Ophthalmology, Johns Hopkins Hospital
KUNKEL, HENRY G., Rockefeller Institute
KUNTZ, ELOISE, Assistant Professor, Vassar College
LANSING, ALBERT I., Professor of Anatomy, Emory University
LAUFFER, MAX A., Professor and Head of Dept. of Biophysics, University of Pittsburgh
LAZAROW, ARNOLD, Professor and Head of Dept. of Anatomy, University of Minnesota
LEVINE, ROBERT P., Assistant Professor, Harvard University
LEWIN, RALPH A., National Research Council, Maritime Regional Laboratory, Halifax, N. S.
LEVY, MILTON, Professor, New York University, Bellevue Medical Center
LLOYD, DAVID P. C., Rockefeller Institute for Medical Research
LOCHHEAD, JOHN H., Professor of Zoology, University of Vermont
LOVE, WARNER E., Associate, Johnson Foundation, Maloney Clinic
LYNCH, WILLIAM F., Professor of Biology, St. Ambrose College
MCCLEMENT, PATRICIA, Research Scientist, Columbia University
MARSLAND, DOUGLAS, Professor of Biology, New York University, Washington Square College
MACCHI, ITALO A., Assistant Professor of Physiology, Clark University
MAZIA, DANIEL, Professor of Zoology, University of California
MEINKOTH, NORMAN A., Associate Professor of Biology, Swarthmore College
MENKIN, VALY, Head of Experimental Pathology, Temple University School of Medicine
METZ, CHARLES B., Associate Professor of Zoology, Florida State University
MILKMAN, ROGER, Teaching Fellow, Harvard University
MILLER, JAMES A., Professor of Anatomy, Emory University
MOORE, JOHN W., Biophysicist, National Institutes of Health
MOUL, EDWIN T., Associate Professor of Botany, Rutgers University
MULLINS, L. J., Associate Professor, Purdue University
NACE, PAUL FOLEY, Associate Professor of Anatomy, New York Medical College
NELSON, LEONARD, Assistant Professor of Physiology, University of Nebraska
O'MALLEY, BENEDICT B., 160 West 88 Street, New York City 24, New York
OOMURA, YUTAKA, Research Associate, Neuropsychiatric Institute, University of Illinois
OSTERHOUT, W. J. V., Member *Emeritus*, Rockefeller Institute for Medical Research

PARKER, JOHNSON, Assistant Professor of Botany, University of Idaho
PASSANO, LEONARD M., Instructor, University of Washington
PIERCE, MADELENE E., Professor of Zoology, Vassar College
PLOUGH, HAROLD H., Professor of Biology, Amherst College
PROSSER, C. LADD, Professor of Physiology, University of Illinois
RAY, CHARLES, JR., Assistant Professor of Biology, Emory University
RAY, DAVID T., Instructor of Zoology, Howard University
ROCKSTEIN, MORRIS, Assistant Professor of Physiology, New York University, Bellevue Medical Center
ROGERS, K. T., Assistant Professor of Zoology, Oberlin College
ROYS, CHESTER, Research Associate, Tufts College
RUBEN, LAURENS NORMAN, Princeton University
RUGH, ROBERTS, Associate Professor of Radiology, Columbia University
SANDEEN, MURIEL I., Assistant Professor of Zoology, Duke University
SCHECHTER, VICTOR, Associate Professor of Biology, City College of New York
SCOTT, ALLAN, Professor of Biology, Colby College
SCOTT, SISTER FLORENCE MARIE, Professor of Biology, Seton Hill College
SCOTT, GEORGE T., Professor of Zoology, Oberlin College
SEAMAN, GERALD R., Associate Professor of Physiology, University of Texas Medical Branch
SHEDLOVSKY, THEODORE, Rockefeller Institute for Medical Research
SLIFER, ELEANOR H., Associate Professor of Zoology, State University of Iowa
SMALL, JEAN E., Graduate Student, Brown University
SOLOMON, SIDNEY, Assistant Professor of Physiology, Medical College of Virginia
SPIEGEL, MELVIN, Research Fellow, California Institute of Technology
SPEIDEL, CARL C., Professor and Chairman, Dept. of Anatomy, University of Virginia
SPYROPOULOS, CONSTANTINE, Assistant Scientist, National Institutes of Health
STARR, RICHARD C., Assistant Professor of Botany, Indiana University
STEELE, R. H., Research Fellow, Muscular Dystrophy Association of America
STEINBACH, H. B., Professor of Zoology, University of Minnesota
STEINBERG, MALCOM S., Graduate Student, University of Minnesota
STEINHARDT, JACINTO, Director, Operations Evaluation Group, Massachusetts Institute of Technology
STEPHENS, GROVER C., Instructor in Zoology, University of Minnesota
STEPHENSON, WILLIAM K., Assistant Professor of Biology, Earlham College
STOKEY, ALMA G., *Emeritus* Professor of Plant Science, Mount Holyoke College
STUNKARD, HORACE W., Professor *Emeritus* of Biology, New York University
SZENT-GYORGYI, A. E., Institute for Muscle Research at Marine Biological Laboratory
TAKAGI, SADAYUKI, Research Associate, Neuropsychiatric Institute, University of Illinois
TASAKI, ICHIJI, Visiting Scientist, National Institutes of Health
TAYLOR, WM. RANDOLPH, Professor of Botany, University of Michigan
TRAUTWEIN, WOLFGANG, Fellow, Johns Hopkins University Medical School
TRINKAUS, J. P., Assistant Professor of Zoology, Osborn Zoological Laboratory, Yale University
TYLER, ALBERT, Professor of Embryology, California Institute of Technology
URETZ, ROBERT B., Instructor in Biophysics, University of Chicago
VINCENT, WALTER S., Instructor in Anatomy, State University of New York College of Medicine
WARNER, ROBERT C., Associate Professor, New York University-Bellevue Medical Center
WEBB, MARGUERITE, Assistant Professor of Physiology, Goucher College
WEISS, PAUL, Rockefeller Institute for Medical Research
WEISZ, PAUL B., Associate Professor of Biology, Brown University
WHITING, P. W., Professor of Zoology, University of Pennsylvania
WICHTERMAN, RALPH, Professor of Biology, Temple University
WILBER, CHARLES G., Chief, Animal Ecology Branch, Chemical Corps Medical Laboratories
WILCZYNSKI, J., Professor of General Biology and Genetics, Lebanese University, Beirut, Lebanon
WILBRANDT, WALTER, Head of Dept. of Pharmacology, University of Berne, Switzerland
WILLEY, CHARLES H., Professor of Biology, New York University, Heights
WILSON, WALTER L., Assistant Professor of Physiology, University of Vermont College of Medicine

YNTEMA, CHESTER L., Professor of Anatomy, State University of New York Upstate Medical Center
YOUNG, R. T., University of Maryland
ZIRKLE, RAYMOND E., Professor of Radiobiology, University of Chicago
ZWEIFACH, BENJAMIN W., Associate Prof. of Biology, New York University, Washington Square College
ZWILLING, EDGAR, Associate Professor, University of Connecticut

Beginning Investigators, 1955

AIELLO, EDWARD, Assistant in Zoology, Columbia University
BRADFORD, WILLIAM DALTON, Medical Student, Western Reserve University School of Medicine
DRAKE, JOHN W., Graduate Assistant, California Institute of Technology
GEIGER, H. JACK, Student, Western Reserve University
KAYE, ALVIN M., Assistant Instructor, University of Pennsylvania
LARIS, PHILIP C., Graduate Student, Princeton University
LAVOIE, MARCEL E., Syracuse University
MCKINNELL, ROBERT GILMORE, University of Minnesota
MORRILL, JOHN B., Florida State University

Research Assistants, 1955

AOTO, TOMOJI, Research Assistant, State University of Iowa
ADAMS, TERRY, Massachusetts Horticultural Society
ALLEN, M. ANN, Indiana University
ALLERAND, C., Albany Medical College
BALABANIS, REBECCA, South Milwaukee, Wisconsin
BASCH, PAUL FREDERICK, University of Michigan
BIRSKY, BILL, Indiana University
BROWN, ROBERT A., Northwestern University
DOWLING, JOHN ALAN, Harvard College
ELLIS, GORDON W., University of California
ERICKSON, JOAN, Radcliffe College
FRIZ, CARL T., University of Minnesota
KAHN, KENNETH, University of Pennsylvania
LACHANCE, LEO E., North Carolina State College
LEFKOWITZ, LEWIS B., Southwestern Medical School
NATHANSON, DONALD L., Amherst College
OBERLANDER, MARCIA I., State University of New York College of Medicine at Syracuse
RAFFERTY, KEEN A., JR., University of Illinois
REGEHR, HULDA, University of Minnesota
SHELBURNE, JAMES CHRISTIE, Emory University
SKINNER, DOROTHY M., Radcliffe College
WATT, DONALD, Columbia University College of Physicians and Surgeons
WILT, FRED, Indiana University
ZIMMERMAN, ARTHUR M., New York University, Washington Square College

Library Readers, 1955

BECK, LYLE V., Associate Professor of Physiology, University of Pittsburgh School of Medicine
BEUTNER, REINHARD H., Des Moines Still College of Osteopathy
BODANSKY, OSCAR, Attending Clinical Biochemist, Sloan-Kettering Institute
CROUSE, HELEN V., Associate Professor, Goucher College
DEAN, HELEN WENDLER, Cambridge, Mass.
DUBOIS, EUGENE, *Emeritus* Professor of Physiology, Cornell University Medical College
EICHEL, HERBERT J., Research Associate in Biological Chemistry, Hahnemann Medical College
FREUND, JULES, Public Health Research Institute of the City of New York
GABRIEL, MORDECAI, Assistant Professor, Brooklyn College

GAFFRON, HANS, Professor of Biochemistry, University of Chicago
GINSBERG, HAROLD S., Associate Professor of Preventive Medicine, Western Reserve University School of Medicine
GLASS, H. BENTLEY, Professor of Biology, Johns Hopkins University
GOLDTHWAIT, DAVID, Associate Member, Dept. of Biochemistry, Western Reserve University
GRANT, PHILIP, Research Associate, Institute for Cancer Research
GREIF, ROGER L., Associate Professor of Physiology, Cornell University Medical College
GUDERNATSCH, FREDERICK, Cornell University Medical College
JOHANSSON, ARNE, Foreign Operations Administration, University of Colorado
JOHNSON, THOMAS N., Assistant Professor of Anatomy, George Washington University Medical School
JONES, SARAH R., Instructor in Zoology, Connecticut College
KABAT, ELVIN A., Professor of Microbiology, College of Physicians and Surgeons
KARUSH, FRED, Associate Professor of Immunology, University of Pennsylvania School of Medicine
KATZ, LOUIS NELSON, Professorial Lecturer in Physiology, University of Chicago
KERSCHNER, JEAN, Assistant Professor, Western Maryland College
KINDRED, JAMES E., Professor of Anatomy, University of Virginia School of Medicine
KINERSLY, THORN, Research Fellow, Yale University School of Medicine
KLEINFELD, RUTH G., Postdoctoral Fellow, National Cancer Institute, Ohio State University
KOLIN, ALEXANDER, Associate Professor of Physics, University of Chicago
LEVINE, RACHMIEL, Chairman, Dept. of Medicine, Michael Reese Hospital
LIPPMAN, HEINZ I., Assistant Clinical Professor, Albert Einstein College of Medicine
LOEWI, OTTO, Research Professor of Pharmacology, N. Y. U., College of Medicine
LOVE, LOIS H., Research Associate, National Research Council
LOEWENFELD, IRENE E., Research Technician, Columbia University
LOWENSTEIN, OTTO, Research Associate, Columbia University
MCDONALD, SISTER ELIZABETH SETON, College of Mount St. Joseph on the Ohio
MILSTEIN, SEYMOUR W., Research Associate, Hahnemann Medical College
NACHMANSOHN, DAVID, Professor of Biochemistry, Columbia University
PEQUEGNAT, WILLIS, Professor of Zoology, Pomona College
PICK, JOSEPH, Associate Professor of Anatomy, N. Y. U. Bellevue Medical Center
RAVIN, ARNOLD W., Assistant Professor of Biology, University of Rochester
ROBERT, NAN L., Instructor in Biological Sciences, Hunter College
ROOT, WALTER S., Professor of Physiology, College of Physicians and Surgeons
ROTHSTEIN, FRED, Hahnemann Medical College
SCHLESINGER, R. WALTER, Associate Member, The Public Health Research Institute of New York
SCHNEYER, LEON H., Associate Professor of Clinical Dentistry, College of Alabama
SCHWABE, LOUISE A., Science Teacher, Kenmore Senior High School
SMELSER, GEORGE K., Professor of Anatomy, Columbia University
SULKIN, S. EDWARD, Professor of Microbiology, Southwestern Medical School
TAUBER, HANS-LUKAS, Assistant Professor, New York University College of Medicine
WAINIO, WALTER W., Associate Professor of Biochemistry, Rutgers University
WAKSMAN, BRYON H., Associate in Bacteriology, Harvard University
WEIDMAN, S., State University of New York College of Medicine at Brooklyn
WHEELER, GEORGE E., Instructor in Biology, Brooklyn College

Students, 1955

BOTANY

AHMADJIAN, VERNON, Clark University
ARCE, GINA, Vanderbilt University
CASHMAN, MARJEAN L., University of Maryland
COURTENAY, WALTER ROWE, JR., Vanderbilt University
COX, SAMUEL F., Vanderbilt University
CROSS, CAROLINE B., Acadia University

EIGER, JOAN V., City College of New York
FREUDENTHAL, HUGO D., Columbia University, College of Pharmacy
GALLOWAY, RAYMOND A., University of Maryland
GATES, JOHN, Cornell University
GREEN, PAUL B., Princeton University
HILFERTY, FRANK, State Teachers College
HOFFER, JOSEPH L., Fordham University
LAMB, IVAN MACKENZIE, Harvard University
OVERSTREET, ROSE ALICE, Indiana University
POKORNY, FRANK J., St. John's University
RADER, PHILIP SCOTT, Middle Tennessee State College
SCHELTEMA, RUDOLF S., Harvard University
WILSON, VANNIE WILLIAM, Morgan State College

EMBRYOLOGY

BAGNARA, JOSEPH T., State University of Iowa
BEARD, ROBERT GORDON, Indiana University
BORODACH, GEROLD N., Brown University
BOURKE, ROBERT SAMUEL, Harvard University
CAREY, FRANCIS GERALD, Harvard University
DAVIS, ROWLAND HALLOWELL, Harvard University
DE LA PAZ, JUSTO, Cornell University
DE TERRA, NOEL, Barnard College
GOLDSTEIN, JOEL B., Haverford College
GREENLESS, JANET LUCILE, University of Wisconsin
HARRIS, PATRICIA J., Yale University
HICKSON, ELIZABETH, Brown University
KNEPTON, JAMES C., JR., Duke University
LAUFER, WILA P., Tufts University
LYSER, KATHERINE MAY, Oberlin College
MCARDLE, EUGENE WILLIAM, Marquette University
MATHESON, GAIL E., Wheaton College
MENDELSOHN, EVERETT, Harvard University
NATHANSON, DONALD LAWRENCE, Amherst College
NEU, HAROLD C., Creighton University
ORELUP, ALETHEA ANN, University of Illinois
PIERCE, PETER G., Colby College
RAFFERTY, NANCY S., University of Illinois
SAGE, JANET KATHLEEN, DePauw University
SCHULTES, SANDRA JEAN, Goucher College
SHOGER, ROSS L., University of Minnesota
SKINNER, DOROTHY M., Radcliffe College
SPENCER, CHARLES DAVID, Wesleyan University
TULL, DADE LOUISE, Vassar College
YOUNG, ROBERT RICE, Yale University

PHYSIOLOGY

BAKER, K. FRANCE, Columbia University
CAZORLA, F. ALBERTO, Instituto NCNAL de Enferme-Dades Neoplasicas, Lima, Peru
CORDEAU, JEAN PIERRE, Université de Montreal
CROCKER, CHARITY S., Instituto de Biofisica, Rio de Janeiro, Brazil
CURRY, GEORGE MONTGOMERY, Harvard University

EKBERG, DONALD ROY, University of Illinois
FERNANDES, JOSE FERREIRA, Medical Faculty, Sao Paulo, Brazil
FLEISHER, JOSEPH H., Chemical Corps, Medical Laboratories
FLEMING, WILLIAM WRIGHT, Harvard University
GOE, DON RICHARD, University of Southern California
GOLDSMITH, TIMOTHY H., Harvard University
GUCCIONE, IGNATIUS, New York University
HERRANEN, AILENE M., University of Wisconsin
HURWITZ, CHARLES, V. A. Hospital, Albany, New York
KEPCHAR, JOHN HOWARD, University of North Carolina
LEFKOWITZ, LEWIS B., JR., Southwestern Medical School
LEVINE, LAURENCE, Wayne University
MACHATTIE, LORNE ALLISTER, University of Buffalo
MAUZERALL, DAVID C., Rockefeller Institute for Medical Research
MENDELSOHN, MARY L., Radcliffe College
MESSINEO, LUIGI, Institute of Zoology of Palermo, Italy
NEUHAUS, FRANCIS C., Duke University
RHODES, WILLIAM C., Johns Hopkins University
ROBERTS, JANE C., University of California, Los Angeles
ROGERS, PALMER, Johns Hopkins University
SMALL, ARLENE MAY, Mount Holyoke College
STEVENS, CARL MANTLE, State College of Washington
URBAN, THEODORE J., Creighton University
WAHBA, ALBERT J., Cornell University
WOHLHIETER, JOHN ANDREW, University of Pittsburgh

ZOOLOGY

ABBOTT, JOAN, Washington University
ADAM, BETTY ROSE, De Paul University
BABCOCK, RICHARD G., University of Michigan
BATES, GRIFFIN MILLER, Hamilton College
BEARD, ROBERT G., Indiana University
BISHOP, JANE ELLEN, Oberlin College
BROWN, EDWARD R., University of Cincinnati
BRUENING, BETTY L., Goucher College
BUTZ, ANDREW, Fordham University
CALI, CARMEN T., Fordham University
CAMOUGIS, GEORGE, Harvard University
CAREY, FRANCIS GERALD, Harvard University
CHRISTENSEN, ALBERT KENT, Harvard University
CHURCH, CHARLES HENRY, JR., Wesleyan University
CRANSTON, MARGARET B., Mount Holyoke College
DAVIS, PETER WRIGHT, Bowdoin College
DICKINSON, WINIFRED, Pennsylvania College for Women
DRURY, GEORGE L., S. J., Boston College
DUNGAN, SHIRLEY R., DePauw University
EAKIN, EDWIN LLOYD, Kenyon College
FABINY, ROBERT JOHN, Marquette University
FASSULIOTIS, GEORGE, New York University
FEELEY, EDWARD J., Fordham University
FINLAY, PETER S., Syracuse University
GATES, JOHN OTIS, Cornell University
GOLDEN, HAROLD J. J., Saint Louis University
GROSS, CHARLES, Harvard College

HALL, WILLIAM H., University of Virginia
HOFSTETTER, SISTER ADRIAN MARIE, Notre Dame University
HORRELL, HARRY CUNNINGHAM, University of Chicago
JOSEPHSON, ROBERT KARL, Tufts University
KINYON, NANCY, Northwestern University
LISA, JOSEPH D., Fordham University
MACHATTIE, LORNE A., University of Buffalo
MARKHAM, ALICE ELINOR, Mount Holyoke College
MAURIELLO, GEORGE E., New York University
MENGES, ELIZABETH V., Smith College
ORR, ANTOINETTE M., Marlboro College
OZBURN, GEORGE W., Ontario College
PANUSKA, JOSEPH ALLEN, St. Louis University
PARSONS, JOHN A., Pennsylvania State University
PASCALE, JANE FAY, University of Chicago
PEACOCK, RONNIE, Earlham College
PICCIANO, ANTOINETTE A., Northwestern University
ROSS, DONALD J., Fordham University
ROUSALL, PAUL G., S. J., Fordham University
SALYERDS, ANNE MARTHA, Agnes Scott College
SCOTT, DIANA F., Swarthmore College
SMITH, SALLY, Vassar College
SPANO, REV. ANTHONY A., Fordham University
SWADER, LAURA LYNN, Drew University
TAYLOR, PETER B., Cornell University
TRUONG, REV. HOANG, Northwestern University
WHITIN, NAVAMONIE, Wellesley College
WISCHNITZER, SAUL, University of Notre Dame
WOODS, JAMES E., DePaul University

ECOLOGY

BING, PETER S., Los Angeles 24, California
CAREY, ANDREW G., Princeton University
COHEN, MATANAH, University of Colorado
DAVIS, ROGER E., University of Wisconsin
DIAL, NORMAN A., University of Illinois
FELITTI, VINCENT JUSTUS, Dartmouth College
FREUDENTHAL, ANITA R., New York University
FREUDENTHAL, HUGO D., Columbia University
HAWS, CLAYTON, Drew University
MCLAUGHLIN, JOHN J., Haskins Laboratories, New York University
MORRILL, JOHN B., JR., Florida State University
NAGLE, MARY ELIZABETH, Clark University
WRIGHT, THEODORE, Yale University

3. LALOR FELLOWS, 1955

CLAYTON, RODERICK K., U. S. Naval P. G. School
GEST, H., Western Reserve University
GREEN, MAURICE, Childrens Hospital, University of Pennsylvania
LEWIN, RALPH, National Research Council, Maritime Regional Laboratory, Halifax, N. S.
MACCHI, ITALO, Clark University
ROTH, JAY, Hahnemann Medical College
WILBRANDT, W., University of Berne, Switzerland

4. Tabular View of Attendance, 1951–1955

	1951	*1952*	*1953*	*1954*	*1955*
Investigators—Total	303	306	310	298	250
Independent	186	172	176	180	162
Under Instruction	28	38	37	20	9
Library Readers	37	49	46	52	54
Research Assistants	52	47	51	46	25
Students—Total	124	123	136	134	148
Zoology	55	55	55	56	56
Embryology	27	23	30	29	30
Physiology	29	27	31	28	30
Botany	13	11	11	12	19
Ecology		7	9	9	13
Total Attendance	427	429	446	432	398
Less persons represented as both students and investigators		2		5	
	427	427	446	427	398
Institutions Represented—Total	158	149	155	136	129
By Investigators	115	92	90	104	95
By Students	43	57	65	32	34
Schools and Academies Represented					
By Investigators	1	1		2	3
By Students		3	1	1	2
Foreign Institutions Represented					
By Investigators	8	7	15	11	8
By Students	3	2	6	13	6

5. Cooperating and Subscribing Institutions, 1955

Cooperating Institutions

Amherst College
American Cancer Society
American Philosophical Society
Brooklyn College
Brown University
Bryn Mawr College
California Institute of Technology
Children's Hospital of Philadelphia
City College of New York
Colby College
College of Mt. St. Joseph on the Ohio
Columbia University
Columbia University, College of Physicians and Surgeons
Cornell University
Cornell University Medical School
Duke University
Elmira College
Emory University
Florida State University
Fordham University
George Washington University Medical School
Grass Foundation
Hahnemann Medical College
Harvard University
Harvard University Medical School
Institute for Cancer Research
Institute for Muscle Research
Johns Hopkins University
Johns Hopkins University Medical School
Lalor Foundation
Eli Lilly and Company
Morgan State College
Mount Holyoke College
National Institutes of Health
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6. Evening Lectures, 1955

June 24
Paul Weiss ..... "Some thoughts and experiments on morphogenesis"

July 1
George Wald ..... "The origin of life—some special problems"

July 8
F. Sjostrand ..... "Ultrastructural organization of retinal receptor cells"

July 15
Bradley M. Patten ..... "Micromoving picture studies of the first heart beats in the beginning of the embryonic circulation"

July 22
F. O. Schmitt ..... "Chemical and structural studies of nerve fibers"

July 29
Walter Wilbrandt ..... "Carrier transport systems and their kinetics"

August 5
F. A. Brown, Jr. ..... "The rhythmic nature of life"

August 12
Arnold Lazarow ..... "Diabetic toadfish; their use in studies on the etiology of diabetes mellitus"

August 19
Kenneth S. Cole ..... "Squid axon excitation"

August 26
George Kidder ..... "Metabolic studies on animal microorganisms"

7. Tuesday Evening Seminars, 1955

July 5

R. P. Levine ..... "Cations in chromosome structure: their relation to the mechanism of crossing over"

Daniel Mazia and Walter S. Plaut ..... "Distribution of parental material in chromosome reproduction"

Max A. Lauffer and Herman Cember ..... "The effects of ionizing radiations on cockroach embryos"

July 12

J. P. Trinkaus and Peggy W. Groves ..... "Differentiation of mixed aggregates of dissociated tissue cells"

R. E. Zirkle, W. Bloom and R. B. Uretz ..... "Chromosome movements and cell division after spindle destruction by irradiation of cytoplasm"

R. B. Uretz, W. Bloom and R. E. Zirkle ..... "Changes in refractive index of irradiated chromosome segments"

July 19

Gerald R. Seaman ..... "Purification and properties of an enzyme system which reversibly cleaves succinate to join two molecules of acetyl coenzyme A"

Jay S. Roth ..... "Ribonuclease inhibition"

Elizabeth Anderson ..... "Metabolism of uridinediphosphoglycosyl compounds"

Alexander Kolin ..... "Some recent experiments on electrokinetic separation of proteins and of microorganisms"

July 26

R. K. Clayton ..... "Tactic responses of purple bacteria"

Warner E. Love ..... "The molecular weight of hemerythrin of *Phascolosoma gouldi* by x-ray diffraction"

Barbara E. Wright ..... "Pteridine coenzymes in one carbon metabolism"

P. F. Scholander ..... "Secretion of inert gases and oxygen by the swim-bladder of fishes"

August 2

Carl C. Speidel ..... "Motion pictures of cellular changes in tadpoles following x-ray irradiation"

Robert Allen ..... "Protoplasmic streaming and amoeboid movement"

S. G. A. Alivisatos ..... "Enzymic synthesis of new dinucleotides by a novel method of biosynthesis"

Paul S. Galtsoff ..... "Structure and function of the ligament of Pelecypoda"

August 9

E. G. Butler and H. F. Blum ..... "Effects of ultraviolet on regenerative activity in urodeles"

S. Hagiwara and T. H. Bullock ..... "Study of intracellular potentials in pacemaker and integrative neurons of the lobster cardiac ganglion"

T. H. Bullock and S. Hagiwara ..... "Further study of the giant synapse in the stellate ganglion of squid"

Charles G. Wilber ..... "Electrocardiogram of the alligator"

August 16

Anna R. Whiting and William E. Murphy	"Differences in response of x-rayed eggs and spermatozoa of Habrobracon to anoxia"
Ralph A. Lewin	"Paralysis in double-mutants of Chlamydomonas"
H. H. Plough and Margaret Roberts	"High frequency of transduction of genes by bacteriophage in Salmonella"
Joyce C. Lewin	"Physiological races of the diatom, *Navicula pelliculosa*"

August 23

Philip Grant	"Some observations on the incorporation of glycine C-14 into amphibian embryos"
Maurice Green	"Fucose metabolism in *Escherichia coli*"
James A. Miller, Jr.	"The potentiation by narcosis of the beneficial effects of hypothermia in asphyxia of the neonatal guinea pig"
W. Wilbrandt and P. Luescher	"Action of a thrombocyte protein on capillary permeability"

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3. ASSOCIATE MEMBERS, 1955

ALDRICH, MISS AMEY OWEN
ALTON, DR. AND MRS. BENJAMIN H.
ANTHONY, MR. RICHARD A.
ARMSTRONG, DR. AND MRS. P. B.
BARBOUR, MR. LUCIUS
BARTOW, MR. AND MRS. CLARENCE
BARTOW, MRS. FRANCIS D.
BARTOW, MR. AND MRS. PHILIP
BELL, MRS. ARTHUR
BRADLEY, MR. ALBERT L.
BRADLEY, MRS. CHARLES CRANE
BROWN, MRS. THORNTON
BURDICK, MR. CHARLES L.
CAHOON, MRS. SAMUEL
CALKINS, MR. G. NATHAN, JR.
CALKINS, MRS. GARY N.
CALKINS, MR. SAMUEL
CARLETON, MRS. WINSLOW

Claff, Mr. and Mrs. C. Lloyd
Clark, Dr. and Mrs. Alfred Hull
Clark, Mrs. Leroy
Clark, Mr. W. Van Alan
Clowes, Mr. Allen W.
Clowes, Mrs. G. H. A.
Clowes, Dr. and Mrs. George, Jr.
Colton, Mr. H. Seymour
Crane, Miss Louise
Crane, Mrs. W. Carey
Crane, Mrs. W. Murray
Crossley, Mr. and Mrs. Archibald M.
Crowell, Mr. Prince S.
Daniels, Mr. and Mrs. F. Harold
Day, Mr. and Mrs. Pomeroy
Draper, Mrs. Mary C.
Elsmith, Mrs. Dorothy
Enders, Mr. Frederick
Ewing, Mr. Frederick
Fay, Mr. and Mrs. Henry H.
Fisher, Mrs. Bruce Crane
Galtsoff, Mrs. Eugenia
Gifford, Mr. and Mrs. John A.
Gilchrist, Mr. and Mrs. John A.
Gildea, Dr. and Mrs. E. F.
Green, Miss Gladys W.
Hamlen, Mr. J. Monroe
Harrell, Mr. and Mrs. Joel E.
Harrington, Mr. and Mrs. A. W.
Harrington, Mr. Robert D.
Houston, Mr. and Mrs. Howard E.
Howe, Mrs. Harrison E.
Janney, Mrs. Walter C.
Jewett, Mr. and Mrs. George F.
Keith, Mr. and Mrs. Harold C.
Kidder, Mrs. Henry M.
King, Mr. Franklin
Koller, Mrs. Lewis
Lawrence, Mr. Milford
Lemann, Mrs. Solen B.
Lobb, Mrs. John
McClintic, Mrs. Guthrie
Marvin, Mrs. Walter T.
Mast, Mrs. S. O.
Meigs, Mrs. Edward B.
Meigs, Dr. and Mrs. J. Wister
Meigs, Miss Mary Roberts
Mellon, Mrs. Richard K.
Miskell, Mr. Joseph B.
Mitchell, Mrs. James McC.
Mixter, Mrs. Jason
Montgomery, Mrs. T. H.
Moore, Mrs. William A.
Mosser, Mrs. Florence M.
Motley, Mrs. Thomas
Newton, Miss Helen K.
Nichols, Mrs. George
Nims, Mrs. E. D.
Packard, Dr. and Mrs. Charles
Packard, Mrs. Laurence B.
Park, Mr. Malcolm S.
Peck, Mr. and Mrs. Samuel A.
Pennington, Miss Anne H.
Redfield, Mrs. Alfred
Reznikoff, Dr. Paul
Riggs, Mrs. Lawrason
Rivinus, Mr. and Mrs. F. Markoe
Rodes, Mrs. Boyle
Root, Mrs. Walter
Rudd, Mrs. H. W. Dwight
Sands, Miss Adelaide G.
Saunders, Mrs. Lawrence
Sinclair, Mr. W. R.
Smith, Mrs. Edward H.
Stanwood, Mrs. F. A.
Stone, Mr. and Mrs. S. M.
Swift, Mr. and Mrs. E. Kent
Swope, Mr. and Mrs. Gerard, Jr.
Swope, Miss Henrietta H.
Tilney, Mrs. Albert A.
Tompkins, Mr. and Mrs. B. A.
Vanneman, Dr. and Mrs. Joseph
Waksman, Mrs. Selman A.
Warbasse, Dr. James P.
Webster, Mrs. Edwin S.
Whitely, Mr. and Mrs. G. W., Jr.
Whitely, Miss Mabel W.
Wickersham, Mr. and Mrs. James H.
Williston, Miss Emily
Williston, Prof. Samuel
Wilson, Mrs. Edmund B.
Wolfinsohn, Mrs. Wolfe

V. REPORT OF THE LIBRARIAN

In 1955, the number of currently-received journals totalled 1554 (51 new). Of these titles, there were 467 (5 new) Marine Biological Laboratory subscriptions; 604 (10 new) exchanges and 179 (13 new) gifts; 74 (2 new) were Woods Hole Oceanographic Institution subscriptions; 183 (12 new) were exchanges and 47 (9 new) were gifts.

The Laboratory purchased 56 books, received 84 complimentary copies (10 from authors and 74 from publishers) and 60 miscellaneous presentations. The Institution purchased 28 titles. The total number of new books accessioned amounted to 228.

By purchase, the Laboratory completed 6 journal sets and partially completed 13 sets. The Institution completed two sets and partially completed two sets. Volumes and numbers received by gift and by exchange completed 13 sets and partially completed 10 sets.

There were 3891 reprints added to the collection, of which 2317 were of current issue.

The Library sent out on inter-library loan 190 volumes and borrowed 57 for the convenience of the investigators. A sum of $246.43 was realized from the sale of duplicate material.

At the end of the year, Dr. E. Newton Harvey presented to the Laboratory his large and valuable collection of 12,000 reprints. Of these, 2500 were added to the shelves, many of which filled in sets, making them of far greater value than heretofore. This gift is greatly appreciated and grateful acknowledgment is herewith conveyed to the donor. The duplicates of the reprints already in the Library's possession will be sent to another library of Dr. Harvey's choosing.

Other gifts of reprints and books were received from Drs. Wm. R. Amberson, Roberts Rugh, Kurt G. Stern and Ralph A. Lewin. Without these generous contributions, the Library would be minus many worthwhile acquisitions.

At the end of the year, the Library contained 65,463 bound volumes and 196,089 reprints.

During the years 1914–1923, Dr. R. P. Bigelow served as Librarian. In the years following, he did not lose interest in the growth and development of the Library and throughout the past ten years, he made many generous and valuable contributions. In keeping with his thoughtfulness, Mrs. Bigelow presented four fine portraits which had hung in her husband's study, namely, those of Brooks, Darwin, Huxley and Lamarck. Through Dr. Bigelow's death, the Library has lost a great friend.

Respectfully submitted,

DEBORAH L. HARLOW,
Librarian

VI. REPORT OF THE TREASURER

MARINE BIOLOGICAL LABORATORY

BALANCE SHEET

December 31, 1955

Investments

Investments held by Trustee:	
Securities, at cost (approximate market quotation $1,500,773)	$ 979,202
Cash	1,441
	980,643
Investments of other endowment and unrestricted funds:	
Pooled Investments, at cost (approximate market quotation $230,368)	213,784
Other investments (note B)	53,076
Cash	9.155
	276,015

Plant Assets

Land, buildings, library and equipment (note A)	2,406,077
Less allowance for depreciation (note A)	955,753
	1,450,324

Current Assets

Cash	36,857
Accounts receivable ($3,596 from U. S. Government)	34,754
Inventories of specimens and Bulletins	61,158
Prepaid insurance and other	8,432
	$2,848,183

Notes:

A—The Laboratory has since January 1, 1916, provided for reduction of book amounts of plant assets and funds invested in plant at annual rates ranging from 1% to 5% of the original cost of the assets.

B—The Laboratory has guaranteed a note of approximately $4,000 of the M. B. L. Club and has pledged as security, therefore, bonds with an original cost of $7,900, included in other investments.

Marine Biological Laboratory

Balance Sheet

December 31, 1955

Endowment Funds

Endowment funds given in trust for the benefit of the Marine Biological Laboratory		$ 980,643
Endowment funds for awards and scholarships:		
Principal	31,738	
Unexpended income	1,377	33,115
Unrestricted funds functioning as endowment		206,378
Retirement fund		35,408
Pooled investments—accumulated gain		1,114
		276,015

Plant Liability and Funds

Mortgage payable on demand, 5%		5,000
Funds expended for plant, less retirements	2,401,077	
Less allowance for depreciation charged thereto	955,753	1,445,324
		1,450,324

Current Liabilities and Funds

Accounts payable	5,767
Unexpended balances of gifts for designated purposes	8,229
Advance payments on research contracts	7,646
Current fund	119,559
	$2,848,183

Marine Biological Laboratory

Statement of Operating Expenditures and Income

Year Ended December 31, 1955

Operating Expenditures

Direct expenditures of departments:	
Research and accessory services	$145,306
Instruction	23,967
Library, including book purchases	30,389
Biological Bulletin	12,684
	212,346
Administration and general	46,988
Plant operation and maintenance	68,974
Hurricane emergency repairs	22,059
Dormitories and dining services	128,460
Equipment purchased from current funds	1,712
	480,539
Less depreciation included in plant operation and auxiliary activities above but charged to plant funds	36,429
	444,110

Income

Direct income of departments:	
Research fees	40,194
Accessory services (including sales of biological specimens, $67,436)	93,230
Instruction fees	17,930
Library fees and income	6,537
Biological Bulletin, subscriptions and sales	15,920
	173,811
Allowance for indirect costs on research contracts	15,747
Dormitories and dining services income	100,768
	290,326
Investment income	73,581
Gifts for current use	121,053
Sundry income	394
Total current income	485,354
Excess of income	$ 41,244

Direct costs on research contracts and reimbursement therefor, $41,311, are not included in operating expenditures or income.

Marine Biological Laboratory

Statement of Current Fund

Year Ended December 31, 1955

Balance January 1, 1955	$ 84,586
Less:	
Amount transferred to unrestricted funds functioning as endowment as of July 15, 1955	6,843
	77,743
Add:	
Provision for uncompleted repairs included in operating expenditures	572
Excess of income over operating expenditures, 1955	41,244
Balance December 31, 1955	$119,559

Marine Biological Laboratory

Summary of Investments

December 31, 1955

	Cost	% of Total	Approximate Market Quotations	% of Total	Investment Income 1955
Securities held by Trustee:					
General endowment fund:					
U. S. Government bonds	$184,206	22.4	$ 178,437	14.4	$ 4,337
Other bonds	285,429	34.8	288,716	23.3	9,079
	469,635	57.2	467,153	37.7	13,416
Preferred stocks	85,788	10.4	80,688	6.5	3,370
Common stocks	265,663	32.4	691,929	55.8	25,148
	821,086	100.0	1,239,770	100.0	41,934
General Education Board endowment fund:					
U. S. Government bonds	48,139	30.4	46,633	17.9	1,113
Other bonds	30,637	19.4	30,425	11.6	997
	78,776	49.8	77,058	29.5	2,110
Preferred stocks	27,281	17.3	26,637	10.2	1,130
Common stocks	52,059	32.9	157,308	60.3	5,350
	158,116	100.0	261,003	100.0	8,590
Total securities held by Trustee	$979.202		$1,500,773		$50,524

MARINE BIOLOGICAL LABORATORY

SUMMARY OF INVESTMENTS—*Continued*

December 31, 1955

	Cost	% of Total	Approximate Market Quotations	% of Total	Investment Income 1955
Investments of other endowment and unrestricted funds:					
Pooled investments:					
U. S. Government bonds	$ 40,349	18.9	$ 38,231	16.6	$ 952
Other bonds	82,428	38.5	81,455	35.4	2,007
	122,777	57.4	119,686	52.0	2,959
Common stocks	91,007	42.6	110,682	48.0	4,382
	213,784	100.0	230,368	100.0	7,341
Other investments:					
Bonds	7,920				330
Investment in General Biological Supply House, Inc.	12,700				17,780
Real estate and mortgage	32,456				
	53,076				18,110
Total investments of other endowment and unrestricted funds	$266,860				$25,451
Total investment income received					75,975
Custodian's fees charged thereto					(254)
Investment income					$75,721

STUDIES ON CROSS-FERTILIZATION AND SELF-FERTILIZATION IN LYMNAEA STAGNALIS APPRESSA SAY[1]

GERTRUDE L. CAIN[2]

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In 1817 Oken obtained fertile eggs from *Lymnaea auricularis* which were reared in isolation during their entire reproductive period. Baudelot (1863) reported both self-fertilization and cross-fertilization in *Lymnaea*. Pelseneer (1920) saw only one polar body extruded from the eggs of *Lymnaea* (three species), and concluded that reproduction in isolated snails was parthenogenetic. However, Colton, (1918) in *L. columella* and Crabb (1927a) in *L. stagnalis* observed two polar bodies and on the basis of their observations concluded that parthenogenesis did not occur. Colton further reported that, although self-fertilization did occur, cross-fertilization was the rule; Crabb reported that cross-fertilization was mechanically impossible (1927b). Seshaiya (1927) concluded from a study of breeding habits of *L. luteola* that both cross- and self-fertilization occurred in this species.

Lang in 1900 claimed that self-fertilization could occur without self-copulation, while Künkel (1908) believed that self-copulation was indispensable to self-fertilization, basing his opinion in part on the observation of self-copulation in *L. auricularis* by Von Baer in 1835. Colton and Pennypacker (1934) reported that self-fertilization in *L. columella* for 93 generations did not decrease the viability of the strain. Boettger (1944), in his survey of the Basommatophora, concluded that self- and cross-fertilization were both common in this order. DeWitt (1954) found the percentage of hatching less in self-fertilized eggs of *Physa gyrina* than in cross-fertilized eggs.

The first genetic proof that both self- and cross-fertilization occur in snails was supplied by Diver, Boycott and Garstang (1925) in a study of the inheritance of inverse symmetry in *L. peregra*. Further proof was obtained in the study of the inheritance of albinism in this snail (Boycott and Diver, 1927). Ikeda and Mura (1934), using shell color as a genetic marker, demonstrated that both self- and cross-fertilization occurred in the land snail, *Bradybaena similaris*.

Bretschneider (1948a, 1948b) investigated the mechanism of insemination and oviposition in *L. stagnalis*. He reported that he had seen sperm balls leaving the seminal vesicle and being swept up the female tract to the hermaphroditic duct, where he assumed fertilization occurred. As additional evidence he reported seeing a complete spermatozoon inside the cytoplasm of an egg still in the duct.

[1] Revised from a dissertation presented to the graduate School of the University of Wisconsin in partial fulfillment of requirements for the degree of Doctor of Philosophy. The author wishes to express gratitude to Professor L. E. Noland for his kindness and assistance during the course of this study. She is also grateful to the General Education Board and the National Medical Foundation Inc. (funds contributed by the National Foundation of Infantile Paralysis) for financial assistance during the time this study was being pursued.

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Holm (1946), in micro-anatomical studies on the reproductive tract of *L. stagnalis*, found a "fertilization pocket" homologous to that found by Meisenheimer (1912) in *Helix pomatia*, but saw no eggs or spermatozoa in it. Perrot (1940) reported a similar structure in *Limax maximus*. Abdel-Malek (1954a, 1954b) saw ova in the corresponding pockets in *Helisoma trivolvis* and *Biomphalaria biossyi*.

The present study was undertaken to determine: (1) the mode of inheritance of albinism in *Lymnaea stagnalis;* (2) the relative frequency of self-fertilization as compared with cross-fertilization in this species; (3) the survival time of spermatozoa after transfer from one snail to another; and (4) the possible location of fertilization of the eggs in this snail.

Materials and Methods

The snails used in this study were obtained from strains that had been maintained in laboratory culture at the University of Wisconsin for over ten years. Culture methods were those of Noland and Carriker (1946).

Mode of Inheritance of Albinism

Although albinism has been found to be inherited as a simple Mendelian recessive in several other gastropods (Boycott and Diver, 1927, in *L. peregra;* Ikeda, 1937, in *Philomycus bilineatus*), it was necessary to verify this for *L. stagnalis* before albinism could be used as a genetic marker in this study.

Accordingly two snails, one from the albino culture and one from the pigmented culture, were isolated until each had deposited at least one egg mass. The offspring from the eggs of the pigmented snail were all pigmented and those from the albino snail were all albinos. The two parent snails were then paired for 42 days. During this time one copulation was observed with the pigmented snail serving as the male. Presumably other such copulations occurred when the snails were not under observation.

After 42 days the two snails were separated. The albino was kept in isolation culture, and its egg masses were collected. The offspring resulting from these egg masses were examined under a binocular microscope six days after hatching. By this time pigment had developed along the mantle collar. Any young not showing pigment were re-examined after another five or six days. Of 885 offspring grown from 28 egg masses laid by the albino snail, 43 were albinos, resulting presumably either from self-fertilization or from previous copulations with other albinos in the original stock culture. The 842 pigmented offspring of the albino parent were clearly the result of fertilization of the eggs of the albino by spermatozoa from its pigmented mate.

Six of these pigmented heterozygotes were selected and isolated before sexual maturity. Each was maintained in solitary culture to insure that only self-fertilization would occur. This self-fertilization is obviously the equivalent of crossing two F_1 heterozygotes. Five or more egg masses were saved from each snail, and the progeny therefrom were grown to the age of "pigment-testing." Of a total of 4909 eggs (49 egg masses) from the six heterozygous snails, 64.7% hatched, and of those that hatched 92.9% survived to be examined for the presence of pigment. Of 2949 thus surviving, 2193 were pigmented and 765 were albinos. On a 3:1 basis the

expected ratio would have been 2212:737. The agreement is close and, on the basis of chi square tests, the difference between the expected and the observed ratio was not significant. It may therefore be concluded with confidence that albinism in *L. stagnalis* is inherited as a simple Mendelian recessive, as in other gastropods.

Prevalence of Self-fertilization

Eighteen albino snails (15 with pedigreed albino parentage and 3 from exclusively albino stock cultures) were paired, each with a homozygous pigmented snail taken from the stock culture which for ten years had shown no albinos. Each of these pairs was kept in a separate dish for varying lengths of time (from 20 to 187 days, depending on the pair). The albino partners were thereafter maintained in isolation culture. The eggs produced by these albinos were saved until the hatching snails reached the "pigment-testing" age to determine the relative numbers of pigmented and albino progeny. It was assumed that the albino progeny resulted from self-fertilization and the pigmented offspring from cross-fertilization. There was a slight possibility that the three snails, taken as adults from albino cultures, might have cross-copulated with other albinos before isolation. Two of these three snails showed 100% pigmented offspring in their first egg mass. The third was never seen to copulate with its pigmented partner, and produced only albino offspring throughout its life. Since all other snails were young (less than 130 days) and since no copulations had been observed in the cultures from which they were taken, the possibility that they had already cross-copulated with other albinos is extremely small.

Of the 18 albino snails paired with pigmented mates, 15 of them after separation produced mainly pigmented offspring during the first month, while three gave only albino progeny. From this it is clear that, when albino and pigmented snails are paired, not only does cross-fertilization occur, but, contrary to the opinion of Crabb (1927b), it is the predominating process.

Ten of the 15 albino snails that produced pigmented offspring after separation from their pigmented mate gave 100% pigmented young in at least one of their egg masses. In five of these it was the first egg mass laid after isolation that gave only pigmented progeny. One showed only albinos in its first egg mass, but by its third egg mass was producing 100% pigmented young. Of the ten snails that gave 100% pigmented offspring in at least one egg mass, four were producing albinos exclusively by the end of their lives. Three others, however, were still producing 100% pigmented progeny in the last egg mass laid before they died. Noland and Carriker (1946) have shown that snails maintained in solitary culture their entire lives frequently will produce more fertile eggs than snails allowed to cross-copulate. It is therefore unlikely that the continued production of pigmented offspring by the three snails mentioned above could have been due to any lack of fertilizing ability on the part of the animal's own sperm.

While cytological tests were not made to eliminate the possibility of parthenogenesis in the case of isolated snails, this seems unlikely because of the almost exact 3:1 ratio obtained in the offspring of the isolated heterozygous snails mentioned earlier in this paper. Had haploid parthenogenesis occurred, a ratio nearer to 1:1 would have been expected. If diploid parthenogenesis had occurred exclusively, only pigmented offspring would have been expected. Moreover, the work of Col-

ton (1918) and Crabb (1927b, 1928) indicated that two polar bodies are extruded by the eggs in *Lymnaea*.

Each of the 18 albino snails mentioned above was kept in isolation culture until its death, with one exception. This snail was discarded after producing nothing but albino offspring in its first five egg masses. The ages of the snails at death, in the 14 cases where it was known exactly, varied from 128 to 465 days. The latter figure represents the oldest snail ever reared in this laboratory.

Longevity of Transferred Sperm

The time elapsing between the separation of an albino snail from its pigmented mate and the laying of its last "pigment-producing" egg gives an approximate figure for the survival time of transferred sperm in the recipient snail. The maximum time found in this study was 116 days. To get some idea about how fast the fertilizing power of transferred sperm is lost, the data obtained from 13 of the 18 snails referred to earlier were combined. Of the five snails not used in the calculations, three (as mentioned above) had not received sperm from their pigmented mate, and two others died too early to give significant data.

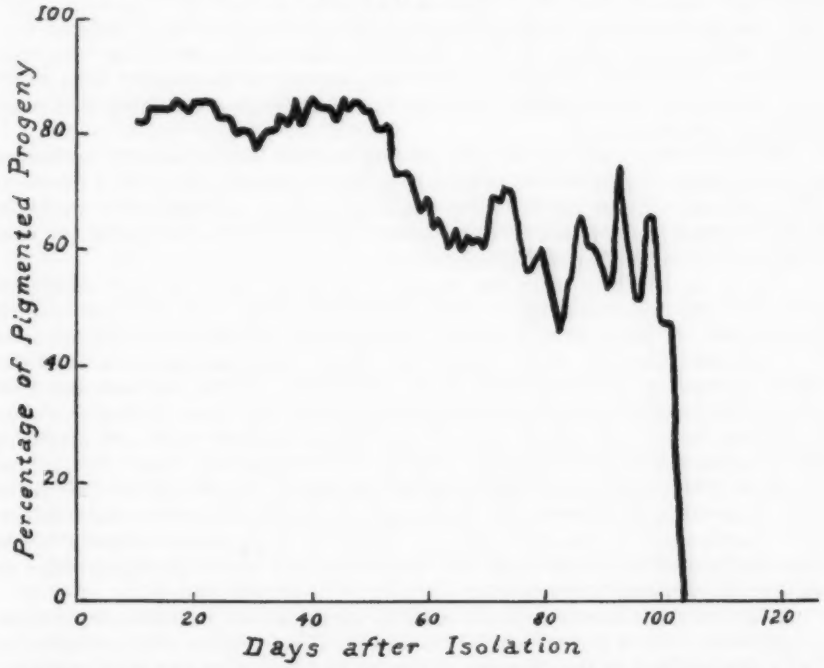

Figure 1. Graph showing rate of decrease in percentage of pigmented individuals in the offspring of albino snails that were isolated after receiving sperm from pigmented snails. (Data obtained from egg masses deposited by the albino after the 102nd day of isolation could not be treated in this graph.)

The data from the 13 remaining snails represented 260 egg masses, containing 15,545 eggs. Of these 72.5% hatched; and of those that hatched, 87.8% survived to the "pigment-producing" age. Figure 1 presents a curve showing the percentage of pigmented individuals in the progeny of the 13 snails plotted against time elapsed since separation from their pigmented mates. The curve represents a moving average, smoothed as follows: each point on the curve represents the total pigmented offspring developing from eggs laid in the 10 days just preceding a chosen point, divided by the total offspring produced from all the eggs laid during the same period. The curve thus represents substantially the percentage of cross-fertilization on successive days following separation.

Examination of the curve shows that in the first 50 days cross-fertilization was very high (over 80%), after which time it gradually fell, dropping rather suddenly near the 100th day. As stated earlier the maximum figure obtained was 116 days. Whether this figure really represents the maximum survival period of the sperm or merely the time at which the supply of transferred sperm was all used up, it is impossible to say.

To arrive at a more exact figure for sperm survival after transfer, it would be necessary (1) to add to the figure obtained (116 days) the time elapsing between the last copulation and the separation of the two partners, and (2) to subtract from the figure the time elapsing between actual fertilization and the laying of the egg. These corrections cannot be made from the data here obtained.

Location of Fertilization

The observations of Meisenheimer (1912), Holm (1946) and Abdel-Malek (1954a, 1954b) suggest that the sperm probably enters the egg in or near the "fertilization pocket." Bretschneider (1948a), however, thinks that fertilization may occur as high up in the reproductive tract as the hermaphroditic duct. (The anatomy of the reproductive system of *Lymnaea stagnalis* is shown in Figure 2.)

If foreign sperm after copulation actually travel up the female tract as far as the hermaphroditic duct, as Bretschneider implies, it would seem likely that they would mix with the sperm of the recipient snail. Then if such a mixture of sperm were later transferred in copulation, it is conceivable that some of the foreign sperm might be passed along to a third snail. This possibility was tested as described below.

Ten albino snails that had never been with pigmented snails were paired with pigmented mates until the albinos were seen to function as females in copulation with those mates. Each of these albinos was then marked with finger nail polish on the tip of the shell and placed with another albino which had never been with a pigmented snail. The pairs were maintained until the marked albino was observed functioning as a male in copulation with the second albino. Eggs were saved from the second albino after isolation, and young snails grown from them. In no case were any pigmented offspring obtained.

This negative result indicates either (1) that the transferred sperm did not reach the level of the hermaphroditic duct in any significant number, or (2) that foreign sperm cannot survive a second passage through the reproductive tract in the process of copulation and later movement up the female tract. Since the foreign sperm had already made such a passage once, it seems a bit unlikely that they could

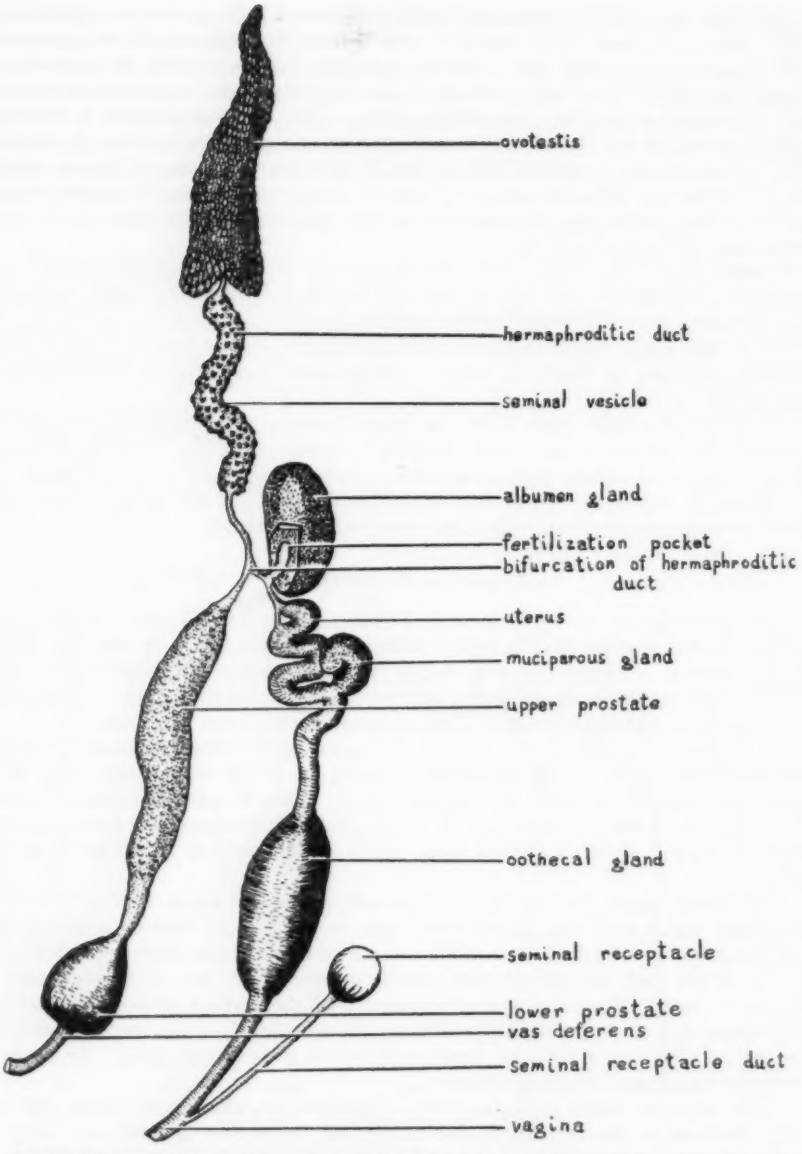

FIGURE 2. Dorsal view of the reproductive system of *Lymnaea stagnalis appressa* (× 40). The vas deferens has been cut away just beyond the point where it joins with the lower prostate gland. The copulatory apparatus is not shown.

not do it again without injury, though they would undoubtedly be greatly diluted by the other sperm with which they were transferred. These results therefore suggest that foreign sperm probably do not travel up the female tract as far as the hermaphroditic duct.

If, as this suggests, fertilization occurs below the bifurcation of the hermaphroditic duct in the oviduct, it must occur very high up in this latter structure, since the albumen and egg shell is laid down around the egg very soon after the egg enters the oviduct (according to Holm and Bretschneider), and no micropyle has ever been found in the snail's egg shell.

If fertilization does not occur above the point of bifurcation of the hermaphroditic duct, self-fertilization could result only after the transfer of sperm by self-copulation. That self-copulation actually does occur has been observed by many workers. Experiments were made to test this possibility.

Even though snails are extremely difficult subjects for surgical experimentation, the intromittent organ was successfully removed in 9 out of 14 cases. These snails continued to lay eggs after self-copulation was no longer possible. The obvious possibility of prior self-copulation could not be excluded. In several snails in which a section of the vas deferens was experimentally removed without subsequent death of the snail, regeneration re-established a connection. The question, therefore, remains unsettled as to whether prevention of self-copulation will also prevent self-fertilization.

The possibility that the seminal receptacle might serve as an activating organ for the sperm was excluded by examination of seminal receptacles removed from snails at different intervals following copulation. Only in those removed within 30 minutes after copulation were motile sperm found, and the motility was less than that of sperm taken from the vas deferens or ovotestis. The problems of the location of fertilization and the function of the seminal receptacle still remain unsolved.

Summary

1. Albinism in *Lymnaea stagnalis appressa* Say is inherited as a simple Mendelian recessive.

2. Cross-fertilization greatly exceeds self-fertilization in snails allowed to cross-copulate.

3. Transferred sperm may remain viable in the body of the recipient snail for as long as 116 days.

4. It is unlikely that foreign sperm are stored as high up in the reproductive tract as the seminal vesicles, since albino snails previously impregnated by pigmented snails and later mated to virgin albinos engender no pigmented offspring in the latter.

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ACCLIMATION OF OXYGEN CONSUMPTION TO TEMPERATURE IN THE AMERICAN COCKROACH (PERIPLANETA AMERICANA) [1]

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The extensive literature that shows metabolic compensation to temperature among marine poikilotherms (see Dehnel, 1955; Segal, 1955; for reviews) has led us to question the generally reported inability to compensate among insects. Scholander, Flagg, Walters and Irving (1953), having compared certain arctic and tropical insects, find no significant metabolic adaptation to temperature. Edwards (1953) generalizes: he proposes that the metabolic response of insects to temperature can be expressed by a single metabolism/temperature curve. Although several examples of insect acclimation are cited by Bullock (1955) he states (p. 320) that "in spite of these cases, it is believed that insects may be relatively poor in ability to compensate."

Of the well documented cases of insect acclimation to temperature, four are either overwintering or in summer sleep (Lühmann and Drees, 1952; Marzusch, 1952); one is aquatic (Sayle, 1928), and one is in pupa (Heller, 1930). Only a single example concerns an active isolated insect (Parhon, 1909).[4]

We have investigated the American cockroach, *Periplaneta americana*, an insect that is active all year, to see whether or not it behaves similarly to marine poikilotherms with respect to temperature adaptation of the oxygen consumption.

Material and Methods

A culture of nymphal and adult cockroaches was obtained from the Riverside campus of the University of California. This culture had been maintained at 27° C. for a minimum of three generations. Neither molting individuals nor adult females nor individuals under approximately 0.3 gram were used in these experiments. The animals were divided into two groups, nymphs and adults, the former being represented by a wide range of instars. Each of the above two groups was subdivided into three groups of randomly selected individuals. There were fifty animals in each of the six groups. One group of nymphs and one group of adults were placed at 10° C. Similar groups of nymphs and adults were placed at 16°

[1] This study was conducted at the University of California, Los Angeles. We wish to thank Dr. T. H. Bullock for making the equipment and space available to us.

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[4] Parker (1930) shows acclimation of growth to temperature in two species of insects (*Melanoplus mexicanus* and *Camnula pellucida*). Thompson (1937) finds that embryos of *Melanoplus differentialis* and *Melanoplus femur-rubrum* kept at lower temperatures (20° C.) show a more rapid heart rate than embryos incubated at higher temperatures (30° and 35° C.) when measured at a series of temperatures from 20° to 35° C.

and 26° C. Cockroaches placed at the latter temperature were at essentially the same temperature as the original stock (26°–27° C.); this permitted them to be used as controls.

In order to eliminate the reported light-controlled diurnal activity rhythm (Cloudsley-Thompson, 1953), the animals were maintained in constant darkness. The cockroaches were weighed after each experiment was completed, as it was felt that the added handling of weighing would stimulate the animals to increased activity.

Each of the six groups of animals was given an initial supply of food, and fresh water was added every other day. It was noticed that the cockroaches at 10° C. did not feed. In order to determine whether non-feeding had any effect on the results, a fresh culture was obtained and the experiment was repeated; this time none of the animals was given food.

Oxygen consumption was measured with the use of Wennesland-Scholander microrespirometers which were submerged in a constant temperature bath controlled to ± 0.5° C. The cockroaches were kept at the acclimation (10° and 16° C.) and control (26° C.) temperatures for three weeks. At the end of each week measurements were made on a nymphal sample from each of the three temperature groups. At the end of the first and third weeks similar measurements were made on adult samples from each of the three temperature groups. All the above oxygen consumption measurements were made at 20° C. Those cockroaches from 16° and 26° C. were kept in the 20° C. bath for one hour before measurements were made. Animals from 10° C. were kept at 15° C. for one hour; the temperature of the bath was then raised to 20° C. After one hour at the latter temperature, their oxygen consumption was measured.

In addition to the above experiments, two samples of nymphs were separated from the original culture (26° C.). The oxygen consumption of those from the first sample was measured over a descending series of temperatures (30°, 25°, 20°, 15° and 10° C.). The first sample was then placed at 26° C. and the other at 10° C. At the end of three weeks the oxygen consumption of animals from the first sample was measured over the same descending series of temperatures. The oxygen consumption of animals from the second sample was measured over an ascending series of temperatures (10°, 15°, 20°, 25° and 30° C.).

Measurements for all experiments were made at fifteen-minute intervals for a period of one and one-half to three hours. On all figures each point represents one animal. The coordinates are log-log and the curves are eye-fitted.

Results

Nymphs. When the oxygen consumption of equal weight animals kept at 10°, 16° and 26° C. is measured at 20° C., it is found that those animals maintained at the lower temperatures show the higher consumption (Fig. 1). The increase in oxygen consumption of animals kept at 10° and at 16° C. occurs within the first week. For the duration of the experiment no further increases were observed. Under the conditions of this experiment it is impossible to compare the time required for acclimation in the two groups; it can be said only that in both it is complete within one week. Because there is no difference in the weekly oxygen consumption values for each group, they are combined for the regression curves in Figure 1.

For purposes of comparison within the three groups of cockroaches, animals with an average weight of 0.6 gram were chosen from the regression curves. This weight was chosen because it falls approximately within the center of the weight range on each of the regression curves. When the oxygen consumption of this 0.6-gram animal is read directly from the graph it is noted that (1) this weight animal acclimated to 10° C. consumes 67 mm.³/gm./hr. (57%) more oxygen than his counterpart acclimated to 26° C., (2) this weight animal acclimated to 16° C. consumes 42 mm.³/gm./hr. (36%) more oxygen than his counterpart from 26° C. and (3) this weight animal from 10° C. consumes 25 mm.³/gm./hr. (16%) more oxygen than his counterpart from 16° C. It is apparent that the animals acclimated

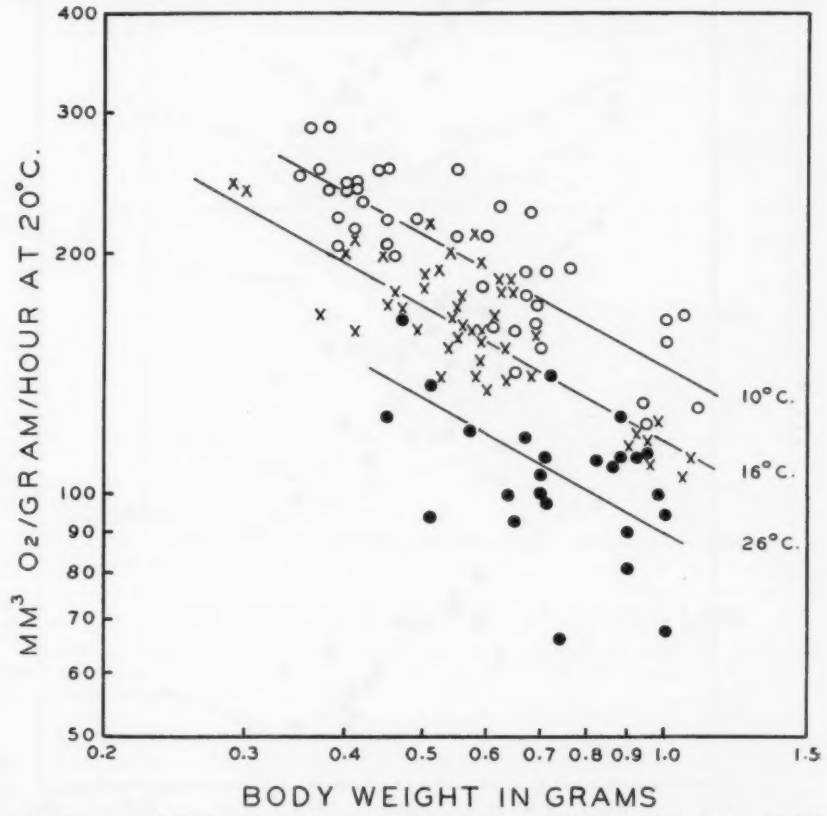

FIGURE 1. Weight-specific oxygen consumption as a function of weight in nymphal *Periplaneta americana*. Animals were kept at 10°, 16° and 26° C. for one to three weeks, and the measurements were made at 20° C. In all figures each point represents the average oxygen consumption for one animal over a period of one and one-half to three hours. Open circles represent 10° C. animals, crosses, 16° C. animals and closed circles, 26° C. ones. The coordinates are logarithmic, and all curves are eye fitted. Results from feeding and non-feeding experiments are combined.

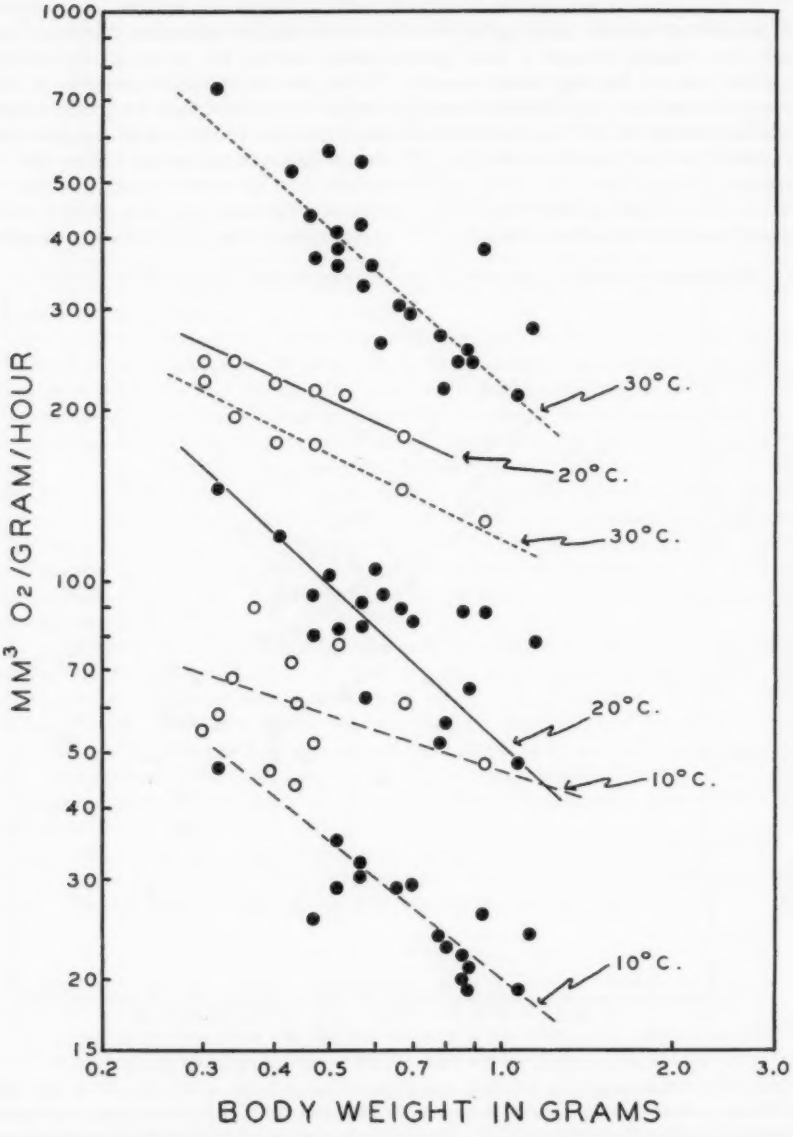

FIGURE 2. Weight-specific oxygen consumption as a function of weight in nymphal *Periplaneta americana* measured over a series of temperatures. Open circles represent cold-adapted animals (10° C.); closed circles represent warm-adapted animals (26° C.).

to 10° C. are responding to the increased distance (° C.) from the control temperature (26° C.) with a further increase in oxygen consumption. The values indicate that a linear relation exists between the increase in oxygen consumption and the decrease in acclimation temperature (4.2 mm.^{3} O_2/gm./hr. increase per degree centigrade drop in temperature).

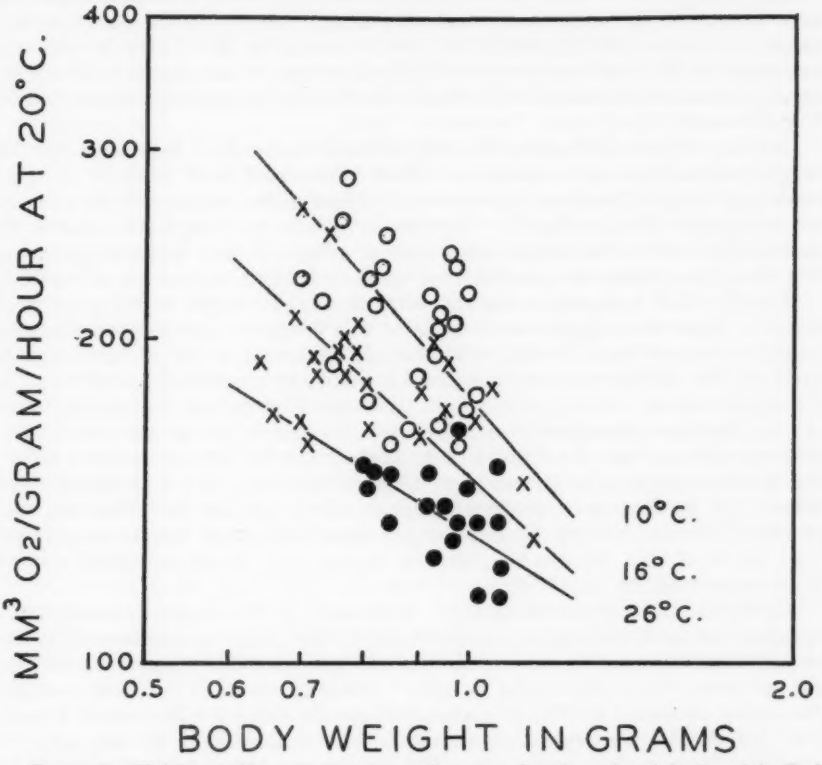

FIGURE 3. Weight-specific oxygen consumption as a function of weight in adult *Periplaneta americana*. Animals were kept at 10°, 16° and 26° C. for one to three weeks, and the measurements were made at 20° C.

Nymphs, rate/temperature experiment. The results of the rate/temperature experiment are presented in Figure 2. The rate values obtained at 15° and 25° C. are omitted to make the graph easier to read. From 10° to 25° C. cold-acclimated nymphs (10° C.) consume more oxygen per gram per hour than equal weight warm-acclimated nymphs (26° C.). The oxygen consumption of the cold-acclimated nymphs is depressed at 30° C., *i.e.*, less oxygen is consumed at 30° C. than at 20° C. Since the curves at 30° and at 20° C. are parallel, the oxygen consumption of large and small nymphs is depressed equally.

At all temperatures the regression lines for the warm-acclimated nymphs are

steeper; the regression lines at the different temperatures for either the warm- or cold-acclimated animals are essentially parallel.

If the curves (Figs. 1 and 2) representing nymphs acclimated to 10° C. and measured at 20° C. are compared, it is seen that the slopes and positions of the curves are the same. However, if a similar comparison is made for the warm-acclimated nymphs, it is found that the oxygen consumption is constantly lower for these animals in the rate/temperature experiment. These animals spent approximately four hours at 30° C., before they were measured at 20° C. It is possible that four hours is sufficient time for the acclimation process to have begun. Therefore, the oxygen consumption at 20° C. is lower than it is for the animals brought directly from 26° to 20° C.

Adults. Adult cockroaches, like nymphal cockroaches, show acclimation of their oxygen consumption to temperature. Those adults kept at 10° and 16° C. consume more oxygen per gram than do equal weight adults kept at 26° C. when all are measured at 20° C. (Fig. 3). As with the nymphs no change was found in the weekly (first and third) oxygen consumption values for each temperature group. Therefore, these values are combined for each of the regression curves in Figure 3.

For the adult cockroaches, animals with an average weight of 0.9 gram were chosen. When the oxygen consumption of this 0.9-gram adult is read from the graph it is noted that (1) this weight animal acclimated to 10° C. consumes 55 $mm.^3/gm./hr.$ (40%) more oxygen than his counterpart acclimated to 26° C., (2) this weight animal acclimated to 16° C. consumes 25 $mm.^3/gm./hr.$ (18%) more oxygen than his counterpart from 26° C. and (3) this weight animal from 10° C. consumes 30 $mm.^3/gm./hr.$ (18%) more oxygen than his counterpart from 16° C. These values suggest that in contrast to the nymphs, a non-linear relationship exists between the increase in oxygen consumption and the decrease in acclimation temperature (2.5 $mm.^3$ $O_2/gm./hr.$ increase per degree centigrade drop in temperature from 26° to 16° C.; 5.0 $mm.^3$ $O_2/gm./hr.$ increase per degree centigrade drop in temperature from 16° to 10° C.).

Comparison of nymphs and adults. Comparison of the oxygen consumption of nymphal and adult cockroaches (Figs. 1 and 3) that have been acclimated to and measured at the same temperatures shows that adult cockroaches consume more oxygen per gram than equal weight nymphs. Although the adult curve representing the control animals (26° C.) is displaced above the curve for the control nymphs (26° C.), the slopes are essentially parallel. With acclimation to 16° and to 10° C. small and large nymphs respond in a like manner and these curves have approximately the same slopes as the 26° C. curve. On the contrary, small and large adults show a differential response to the temperatures of acclimation (with extrapolation, the curves in Fig. 3 would intersect to the right). Small adults are responding to the decreased temperatures of acclimation with a greater increase in their weight-specific oxygen consumption than are large adults. Small adults are therefore doing a better job of acclimating than large adults and all sizes of nymphs are doing a better job than all sizes of adults.

Discussion

The object of this investigation was to see if the insect *Periplaneta americana* could acclimate its metabolic activity to temperature. However, we would first like

to discuss an additional observation. We have found that adult cockroaches are living at a faster metabolic pace than are nymphs of approximately the same weight (see Figs. 1 and 3). Batelli and Stern (1913) showed that at all temperatures from 20° to 40° C. fly imagines consume more oxygen per unit body weight than did larvae. Similarly, it was found by Ludwig (1931) that the weight-specific oxygen consumption of adult Japanese beetles, *Popillia japonica,* was greater than that of the larvae. Referring to the Holometabola, Wigglesworth (1950, p. 413) has stated that "metabolism at a given temperature is generally much higher in the adult than in the larva and higher in the larva than in the pupa." Wigglesworth attributes this difference to the increased activity metabolism of the adult. Many investigators will speak of the typical "U-shaped" respiratory curve during the metamorphosis of holometabolus insects (see Edwards, 1953, for references).

The available data for the Hemimetabola (the insects in which there is little or no change in shape during ontogeny) are much less than for the Holometabola. Edwards (1953) presents a curve showing the change in weight-specific oxygen consumption from egg deposition through early adulthood of the milkweed bug *Oncopeltus fasciatus.* Within a few days after the last molt, adults consume more oxygen per gram per hour than do last instar nymphs. If the oxygen consumption during molt is ignored, then the curve resembles a flattened "U-shape." In this study early adult and late nymphal *Periplaneta americana* show a similar relationship. With increasing size of the adult, the characteristic fall in weight-specific oxygen consumption is observed.

We do not know what makes possible the elevated metabolic activity of the early adult cockroaches; we have not observed a difference in locomotor activity that would account for it. Perhaps the elevation in rate is a consequence of the metamorphosis from nymph to adult. A similar suggestion was offered by Groebbels (1925) to account for the increase in metabolic rate found during metamorphosis of *Rana* tadpoles.

Contrary to the generalized statement of Edwards (1953) that insects do not compensate metabolically to temperature, both nymphs and adults of *Periplaneta americana* adapted to 10° C. consume more oxygen, per animal and per gram, than equal weight control animals adapted to 26° C. when measured at the same temperature.

Lühmann and Drees (1952) and Marzusch (1952) show temperature adaptation in four species of insects, two of which are overwintering (the potato beetle, *Leptinotarsa decemlineata,* and the leaf beetle, *Phytodecta rufipes*) and two in summer sleep (the potato beetle, *Melasoma populi,* and the leaf beetle, *Galeruca tanaceti*). These investigators are unable to show temperature adaptation during the active feeding period. Lühmann and Drees have suggested that the compensatory response is masked by the high metabolic activity associated with feeding. We believe that if such a response can be demonstrated at any given time, it does seem reasonable to expect this ability to be present at all times. This expectation is borne out by *Periplaneta americana,* which is active and feeds all year. Therefore, it is difficult to understand why this compensatory response appears only in these insects under conditions of winter and summer sleep. It would be well to note that one species of leaf beetle (*Chrysomela haemoptera*) shows no adaptation even though its metabolic level was depressed during the summer sleep (Lühmann and Drees, 1952).

Previously, cold- and warm-adapted groups have been compared at a given temperature or between temperatures by arbitrarily choosing a weight and determining the oxygen consumption for each group. It is also profitable to choose an arbitrary rate of oxygen consumption and determine the approximate weight of animal in each group for which this rate is obtained (Figs. 1 and 3). As a generalization, a large cold-adapted cockroach consumes about as much oxygen as a small warm-adapted one. For example, a 0.4-gram nymph (26° C.), a 0.7-gram nymph (16° C.) and a 0.9-gram nymph (10° C.) when measured at 20° C. consume equal amounts of oxygen per unit weight. Similarly, it is possible to determine the temperatures at which cold- and warm-adapted roaches consume the same amount of oxygen (Fig. 2). On this basis, cold-adapted animals consume at 15° C. slightly more oxygen than warm-adapted animals consume at 20° C.

Sayle (1928) tested the effect of low temperature on carbon dioxide production of dragon fly nymphs (*Aeschna umbrosa*). She lowered the temperature from 22° C. to 13° C. (three days at 17° C. and three days at 13° C.) and found that carbon dioxide production was about the same at the lower temperature as the initial production at 17° C. after the first day. The major portion of acclimation of these nymphs was evident within forty-eight hours. It is not unreasonable to expect that the rate of acclimation in *Periplaneta americana* is equally as rapid since no further change was evident after six days. In addition, animals measured at 20° C. after spending a number of hours at 30° C. consume less oxygen than animals measured at 20° C. directly from 26° C. Such time courses as found in these animals compare favorably with that shown for other species (Behre, 1918; *Planaria dorotocephala;* Roberts, 1952, *Pachygrapsus crassipes;* Segal, 1955, *Acmaea limatula*).

Bullock (1955) has thoroughly reviewed the known cases of acclimation to temperature at the several levels of organization (molecular, cellular, tissue and organ system). He does cite several negative instances in which animals fail to show acclimation. However, the evidence from widely divergent groups, involving different physiological systems, suggests to us that compensatory responses to environmental stresses are inherent components of protoplasmic systems. Negative cases as cited by Bullock (1955) do not invalidate this idea. Such instances suggest to us that animals, in which no acclimation was found in the particular physiologic system studied, might show compensation to stress in another system or at a different level. Compensatory responses to temperature are most often described, but other environmental parameters (osmotic pressure, drugs, oxygen tension; see Prosser, 1955) equally as important may evoke such adaptation. If this phenomenon is a universal component of living systems and permits animals to assume degrees of environmental independence, it goes far to explain their survival and distribution. Within limits it accomplishes the same results as homiothermism accomplishes for the warm blooded animals.

Summary

1. Oxygen consumption has been studied in cultures of nymphal and adult cockroaches, *Periplaneta americana,* that have been maintained at two experimental temperatures (10° and 16° C.) and the control temperature (26° C.) for a period of one to three weeks.

2. It has been shown that the oxygen consumption of equal-weight nymphs when measured at 20° C. is higher in animals that have been maintained at the lower temperatures.

3. Comparison of cold- (10° C.) and warm-adapted (26° C.) nymphs when measured at a series of temperatures (10° to 25° C.) demonstrates that cold-acclimated animals consume more oxygen per gram per hour than equal weight warm-adapted ones.

4. Adult cockroaches show acclimation of their oxygen consumption to temperature. However, there is a differential response with respect to size; small adults acclimate to a greater degree than large ones. Further, all sizes of nymphs show a greater degree of acclimation than all sizes of adults.

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NEUROSECRETORY CELL TYPES AND THEIR SECRETORY ACTIVITY IN THE CRAYFISH [1, 2]

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It is now well known that physiologically active substances are produced in neurosecretory cells located throughout the nervous systems of crustaceans (Bliss, 1951, 1952, 1953; Bliss, Durand and Welsh, 1954; Bliss and Welsh, 1952; Carlisle, 1953; Enami, 1951; Passano, 1951a, 1952, 1953). Furthermore, the neurosecretory cells are distributed as distinct groups (Bliss, Durand and Welsh, 1954; Enami, 1951), at least in the eyestalk and brain. Relatively little is known about the specific localization of the sources of the neurohormones affecting particular physiological processes; however, Passano (1951a, 1951b, 1952, 1953) has shown that the x-organ in crustaceans produces a substance that is capable of inhibiting molt.

Neurosecretory cells have been described for the x-organ (Bliss, 1952; Bliss, Durand and Welsh, 1954; Bliss and Welsh, 1952; Carlisle and Passano, 1953; Enami, 1951; Passano, 1953); but, with the exception of Enami's work on *Sesarma* (1951), there is little information concerning the different types of neurosecretory cells present in crustaceans. Furthermore, there is no cytological evidence available to indicate which of the different neurosecretory cell types are involved in the physiology of molt. It is apparent that work along these lines is needed, particularly in view of the fact that cytological differences in cell types often go hand in hand with differences in function.

The present paper will be concerned with a histological study of the neurosecretory system of the crayfish, *Orconectes virilis* (formerly *Cambarus virilis*) in relation to the molting cycle.

Materials and Methods

1. *Animals*

The animals used in this study were mature males, approximately five centimeters in carapace length, all collected from Hobb's Brook Reservoir, Lincoln, Mass., in the summer of 1954. Mature crayfish were collected on the dates shown in Table I. With the exception of May animals, which had been kept in the laboratory for three to four months and fed weekly on clam and fish, eyestalks and brains were removed and fixed on the same day the animals were collected.

[1] This work constitutes a portion of a thesis submitted in partial fulfillment of the requirements for the Ph.D. degree from Harvard University. The writer wishes to express his sincere thanks to Professor John H. Welsh under whose direction this work was carried out.

[2] The preparation of the manuscript was aided by a grant from the Research Council, Rutgers University.

2. Dissections

All dissections were performed in crayfish perfusion fluid (van Harreveld, 1936). A pair of fine iridectomy scissors, jeweler's forceps and cuticle scissors were used in the dissections.

Eyestalk. The eyestalk was removed by cutting the pedunculus lobi optici with a pair of small cuticle scissors. Next, the chitinous exoskeleton was cut the full length of the eyestalk on each side. The eyestalk was then pinned ventral side down by means of size 0 insect pins in a Syracuse watch glass, half filled with paraffin and containing crayfish perfusion fluid. The remainder of the dissection was carried out with the aid of a binocular dissecting microscope.

After a cut was made across the dorsal half of the retina, the proximal end of the top half of the exoskeleton was lifted and the hypodermis was carefully scraped from the exoskeleton. Great care was taken in this step to prevent excess stretching of the nerve tissue.

The cut end of the pedunculus lobi optici was grasped with fine forceps, and the eyestalk contents were separated from the underlying exoskeleton. The whole content of the eyestalk was then placed in a vial containing fixative. With practice, this procedure could be accomplished within two to three minutes. Excellent fixation was obtained in all cases.

Brain. The head of the animal was removed by a cut just posterior to the brain and mouth. The exposed parts were immediately rinsed thoroughly with perfusion fluid to remove any stomach contents, pieces of hepatopancreas, or urine released after puncture of the bladders. Frequent changes of the perfusion fluid were made throughout the dissection. The rostrum of the animal was next inserted in a piece of modeling clay in such a manner that the open end of the head was facing up. In this way, the animal's head served as a miniature dissecting vessel. The remainder of the procedure was carried out with the aid of a dissecting microscope.

After removal of the stomach, pieces of hepatopancreas, and green glands, the brain was rinsed thoroughly with perfusion fluid. All nerves leading from the brain and the connective tissue sheath surrounding the brain were cut away, and the brain was placed in a vial containing fixative. The brain was lifted by means of the circumoesophageal connectives. This procedure required about three to four minutes.

3. Histological procedure

The fixatives employed in this study were Helly's fluid (fixing time, eight hours) and Bouin's plus one per cent calcium chloride (fixing time, twenty-four hours). Tissues were dehydrated in alcohol, cleared in cedar oil and embedded in Tissuemat (melting point 56–58° C.). Sections were cut at 6 μ and stained with aldehyde fuchsin (Gomori, 1950) according to the schedule of Halmi (1952), but with modifications by Dawson (1953). This procedure involved a permanganate oxidation prior to staining and will be referred to in the text as PAF. Sections were also stained with chrome-alum-hematoxylin-phloxin (Gomori, 1941) as adapted by Bargmann (1949). This technique is referred to in the text as CHP.

4. *Cell counts*

A study of cell types revealed that secretory material was present as small granules or droplets within the cells. The secretory activity of a group of cells could be judged, therefore, by counts of cells which appeared histologically to be in a given stage of the secretory cycle.

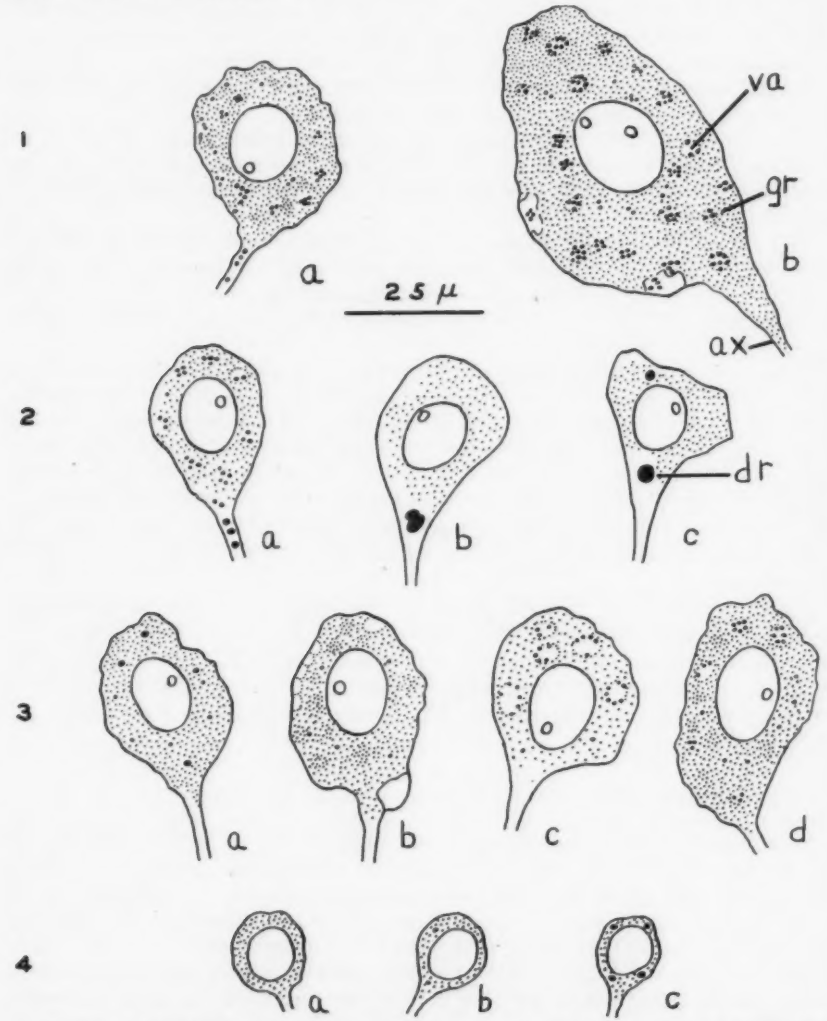


FIGURE 1. Drawings of neurosecretory cell types in the eyestalk and brain of the crayfish. Numbers along the left column indicate cell types. Letters indicate cells in successive stages of the secretory cycle. ax, axon; dr, droplet; gr, granule; va, vacuole.

Type 1 cells of the x-organ, in a stage of the secretory cycle similar to that shown in Figure 2, were counted. These cells are large enough so that they can be recognized from section to section and were counted only when the nucleus was included in the section. In this way no cell could be counted twice.

Type 2 cells in the x-organ were also counted. In this case, cells which contained both a nucleus and a secretory droplet (Fig. 1, Cells 2b, c) in the same section were counted. The nuclei of these cells are small enough so that a section near the center of the nucleus would be present only once per cell. This method of counting resulted in minimum counts of the cells in that particular stage of the secretory cycle. Type 2 neurosecretory cells as shown in Figure 1, Cell 2a, were not counted. The marked uniformity of cell counts during all months except May and June indicates that consistent results can be obtained in this manner.

This method of counting could not be applied to the other neurosecretory cell groups because the secretory material in those groups is freely distributed throughout the cytoplasm in the form of fine granules (see below).

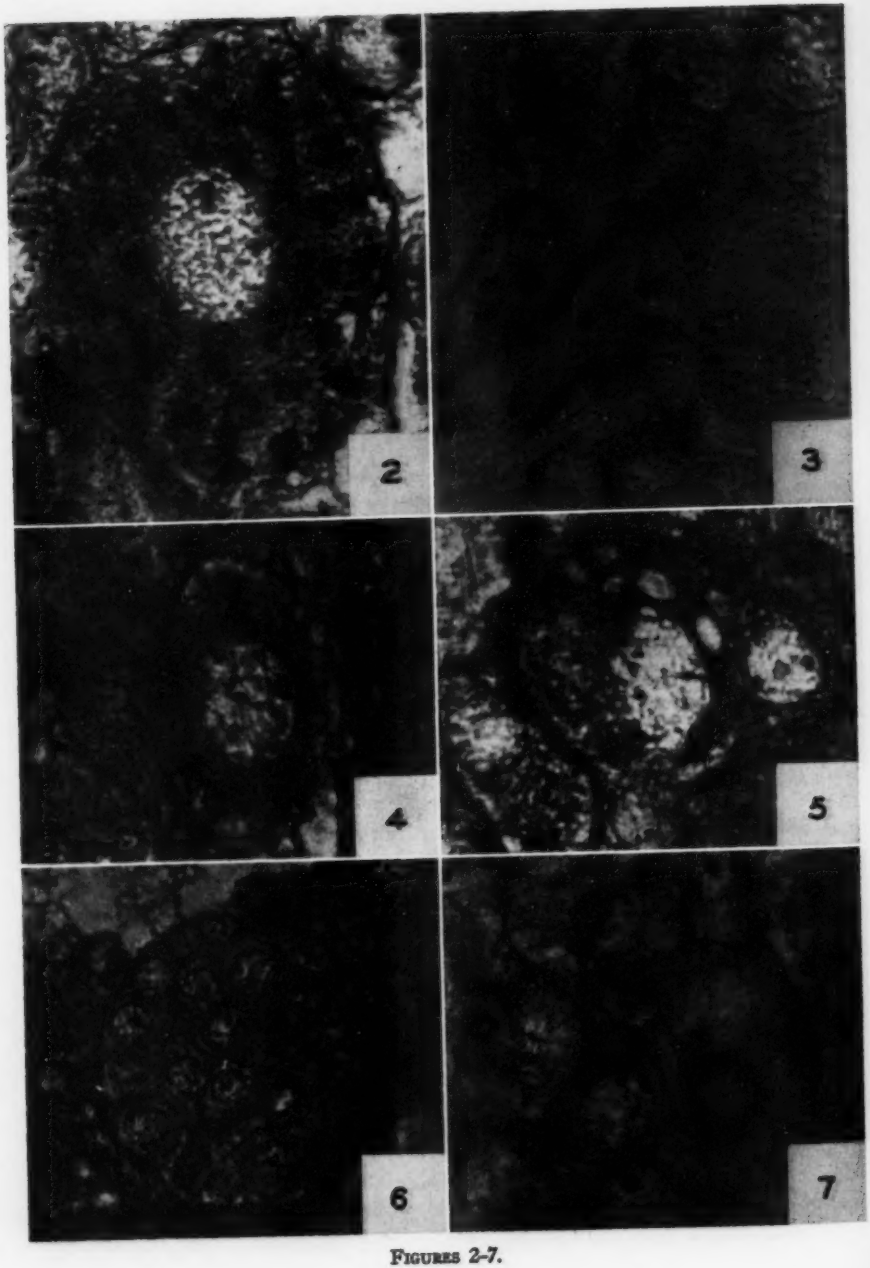
Results

Studies of serial sections of eyestalks and brains, stained with CHP and PAF, have revealed the presence of large groups of cells (see also Bliss, Durand and Welsh, 1954) that are histologically different from the hundreds of ordinary ganglion cells present throughout the eyestalk and brain. These cells are always larger than the ordinary ganglion cells. Most of them possess large nuclei, have abundant cytoplasm, and are characterized by the presence in the perikaryon and axon of droplets of a material which stains conspicuously with aldehyde fuchsin and chrome-hematoxylin. Not all the cells have the same appearance as to the quantity and size of these droplets. These characteristics lead to the conclusion that the cells are neurosecretory cells as defined by E. Scharrer and B. Scharrer (1945) and described in a great variety of animals by numerous authors (see especially Scharrer and Scharrer, 1954; Gabe, 1954).

There appear to be four neurosecretory cell types (Fig. 1) found in the eyestalk and brain of the crayfish, *O. virilis*. Size, general shape of the cell body, presence or absence of vacuoles in the cytoplasm and the appearance of the secretory product were used as the main criteria in separating the cell types. Since large numbers of the cells were found to form more or less distinct subdivisions of larger units in the case of two cell types, and possessed a fairly uniform set of the characteristics listed above, it is believed that the cells are truly of different types and have not been confused with various stages of the secretory cycle present in a given cell type. The only cells that others might possibly find difficult to recognize are those similar to Type 1 (a) and Type 3 (d) (Fig. 1).

Cell types

Cell Type 1. The distribution of this cell type is somewhat limited; it is most numerous in the x-organ and lies as a distinct subgroup in the most distal portion of the x-organ. The cell bodies are large, 40–60 μ in length, possess much cytoplasm and contain a large nucleus, 15 μ in diameter. In the material used in this study, Type 1 cell bodies have extremely irregular outlines which are very likely caused by shrinkage during fixation (Fig. 1). The nucleus may often contain two and some-



FIGURES 2-7.

times three nucleoli. Moreover, nucleoli are usually peripheral, lying against the nuclear membrane. Further, not all of the cells show the presence of large amounts of secretory material at any one time. This, however, is to be expected, for apparently some cells are at the peak of their secretory processes while others are in a quiescent state. The secretory product consists of a great number of aggregations of small (0.5–1.0 μ) granules that stain with aldehyde fuchsin (Figs. 1 and 2). It frequently appears as though the aggregations are located on the surface of small clear spaces in the cytoplasm. In cells that do not contain large amounts of secretory material, aggregations may not be present. In these cases secretory material is scattered in the cytoplasm as fine granules about the size of those that make up the aggregations. The cytoplasm is generally flaky in appearance and, in cells containing many granules, may sometimes be stained a red-purple by the PAF technique. However, many of the cells do not show this cytoplasmic staining; this is probably because the cells are in different stages of the secretory cycle.

In some sections, secretory material may also be seen at a point where the axon leaves the cell body and along the axon for a short distance. When it is found along the axon, the secretory material appears as a number of small granules strung out along the axon. Farther from the cell bodies, though, it appears to consist of a more finely divided suspension somewhat dispersed in the axons.

Cell Type 2. This cell type is a smaller cell which is also restricted in its distribution. These cells are arranged in the proximal part of the x-organ as a cluster of grapes as described by Hanström (1931). The cell body measures about 30 μ in length and is slightly narrower, 20–25 μ, than it is long (Fig. 1). It possesses a large nucleus, but none has been observed to contain more than one nucleolus. The nucleolus here is also near the nuclear membrane. The cytoplasm is somewhat vacuolated, although the vacuoles appear to be a result of fixation; they do not possess any definite shape. In February, these cells possess small vacuoles with granules of secretory material located peripherally. Some cells in February have granules contained within vacuoles. At other times of the year the material is present as quite large, 4 μ, distinct droplets (Figs. 1 and 9) as contrasted with the granules present in Type 1 cells. The droplets, usually one or two per cell, are almost always round and are usually located in the axon hillock or in the axon. Sometimes many drops may be seen along the bundles of axons as they leave the cell group (Fig. 3).

FIGURE 2. Type 1 neurosecretory cell in advanced stage of the secretory cycle. Note the aggregations of granules. Cells with this appearance were counted as indicating the secretory activity of this cell type. Bouin's plus 1% calcium chloride; permanganate-aldehyde-fuchsin, 1300 ×.

FIGURE 3. Type 2 neurosecretory cell containing many droplets of secretory material in its axon hillock. Bouin's plus 1% calcium chloride; permanganate-aldehyde-fuchsin; 1300 ×.

FIGURE 4. Type 3 neurosecretory cell showing peripheral arrangement of vacuoles (top of photograph). Bouin's plus 1% calcium chloride; permanganate-aldehyde-fuchsin; 1300 ×.

FIGURE 5. Type 3 neurosecretory cell. Note centrally located vacuole with granules of secretory material located on the surface. Bouin's plus 1% calcium chloride; permanganate-aldehyde-fuchsin; 1400 ×.

FIGURE 6. Group of Type 4 neurosecretory cells in the eyestalk. Note scanty cytoplasm and scarcity of secretory material. Bouin's plus 1% calcium chloride; permanganate-aldehyde-fuchsin; 700 ×.

FIGURE 7. Group of Type 4 neurosecretory cells in the brain. Note large content of secretory material. Bouin's plus 1% calcium chloride; permanganate-aldehyde-fuchsin; 1200 ×.

Cell Type 3. This cell type is distributed freely throughout the neurosecretory cell groups in the eyestalk and brain with the exception of the x-organ. These cells are, on the average, slightly larger than the Type 2 cells (Figs. 1, 4 and 5). They are generally tear-drop shaped, although not as distinctly so as the Type 2

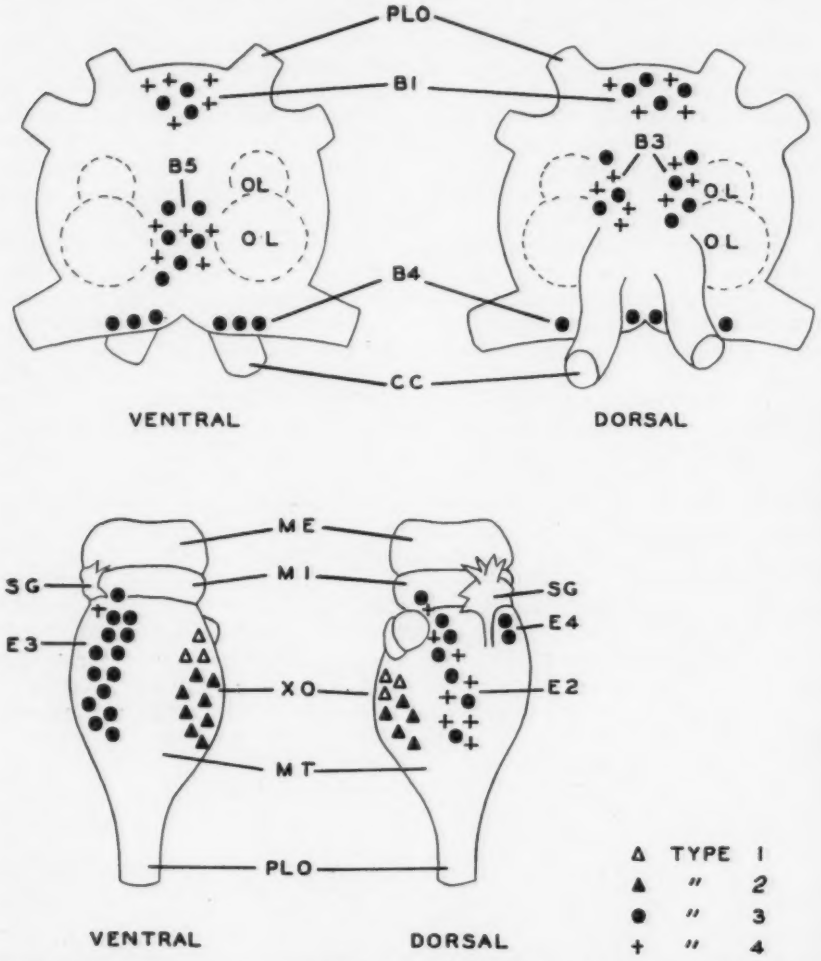


FIGURE 8. Diagrammatic representation of the distribution of neurosecretory cell types in the brain and eyestalk of the crayfish. Compare with Enami's Figure 11 (1951). B1 through B5 designate groups of neurosecretory cells in the brain. E1 through E4 designate groups of neurosecretory cells in the eyestalk. CC, circumoesophageal connectives; ME, medulla externa; MI, medulla interna; MT, medulla terminalis; OL, olfactory lobes; PLO, pedunculus lobi optici; SG, sinus gland; XO, x-organ.

cells. Two characteristics distinguish these cells from the Type 2 cells. The first characteristic is the nature of the vacuoles. The vacuoles are rather large, up to 7 μ, and most of the time may be seen around the periphery of the cell (Figs. 1 and 4) although sometimes they may be located more centrally in the cytoplasm (Figs. 1 and 5). The vacuoles are sharply delimited from the cytoplasm. In this way they are markedly different from those usually found in Type 2 cells. The second characteristic is the appearance of the secretory product. Thus, in the Type 3 cells, the secretory product consists of fine granules which are never clumped in as large numbers as they are in the Type 1 cells (Fig. 2). Furthermore, secretory material is never present in the form of large droplets as it is in Type 2 cells. Granules are scattered, apparently at random, throughout the cytoplasm or they may be found on the surface of vacuoles or, sometimes, as a small drop in the center of one of the vacuoles (Fig. 1). Vacuolated cells of this type are sometimes found to contain no signs of secretion.

Cell Type 4. Cells of this type are located in all neurosecretory cell groups of the eyestalk and brain except the x-organ and group E3 (Fig. 8). The Type 4 cells are small, about 13 μ in diameter, possess a small nucleus, 10 μ in diameter, and, as is obvious from the measurements, very little cytoplasm (Figs. 1 and 6). They are classified as neurosecretory cells since preliminary studies show that, under certain conditions, some of the cells undergo changes in the amount of secretory material they contain (Fig. 7). Furthermore, they are similar to the gamma neurosecretory cells described by Enami (1951) and are found only within the neurosecretory cell groups. Generally they show little sign of secretory activity but differ from the ordinary ganglion cells of the eyestalk in that they possess more cytoplasm and cell boundaries which are easily demonstrated by the techniques used in these studies. The boundaries of the ordinary ganglion cells are extremely difficult to detect with these techniques.

The distribution of neurosecretory cell types is shown in Figure 8. It should be noted that certain cell groups of the crayfish differ in their distribution from that reported in a previous account (Bliss, Durand and Welsh, 1954). The earlier account is essentially correct. However, groups B2 and B3 of the earlier account most likely constitute one group of cells. The group was previously reported to lie lateral to the olfactory lobes. Actually it is located medial to the olfactory lobes on the lateral side of the main mass of fibers of the brain. This distribution of neurosecretory cells brings the neurosecretory system of the crayfish into fairly close agreement with that of *Sesarma* (Enami, 1951).

Secretory activity

No published observations on the normal molting cycle of *O. virilis* are available. However, the following information, although incomplete, shows that there is a single molting time per year for crayfish of the size and sex used in this study. All animals collected on June 28 were soft; the cuticle was parchment-like. Considerable resorption of calcium had occurred from all parts of the exoskeleton and especially from the ischiopodite of the cheliped. Further, all of the animals possessed well developed gastroliths, about 3 mm. in diameter, contained within the gastrolith sac.

Similarly, all animals collected on July 23 were soft; their exoskeletons were

thin and parchment-like, but none possessed any signs of gastroliths. All animals collected on August 14 had hard exoskeletons and showed no signs of an approaching molt.

Since the gastroliths disappear very shortly after molt, the observations indicate that these animals had molted some time between June 28 and July 23. Furthermore, the observations show that the adult male animals used in this study were highly synchronized in their molting period, for none appeared to be approaching a molt on any date after June 28.

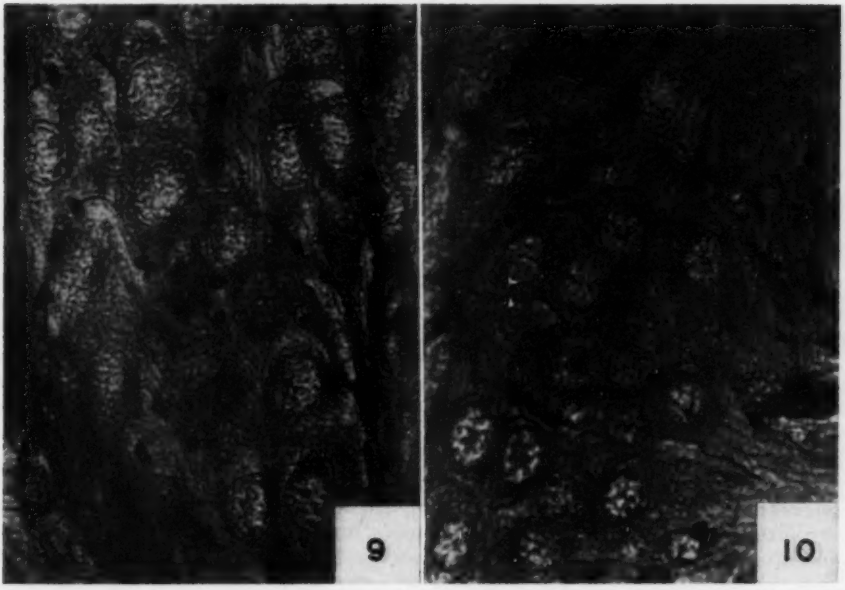


FIGURE 9. Type 2 neurosecretory cells in the x-organ of a crayfish just prior to molt. Note the large number of secretory droplets. Bouin's plus 1% calcium chloride; permanganate-aldehyde-fuchsin; 700 ×.

FIGURE 10. Type 2 neurosecretory cells in the x-organ of a crayfish shortly after molt. Note the lack of secretory material. Bouin's plus 1% calcium chloride; permanganate-aldehyde-fuchsin; 700 ×.

For the greater part of the year, the number of cells that contained secretory material was remarkably constant. However, a striking increase in the number of Type 2 cells that contained droplets of secretory material took place some time before May 6. From Table I it will be observed that more than twice as many Type 2 cells contained secretory droplets on May 6 and June 28 (Fig. 9) than at any other time of sampling (Fig. 10).

Table I shows that the number of Type 1 cells that contained secretory material did not change appreciably throughout the year.

The other neurosecretory cell groups in the eyestalk were examined carefully, but no apparent histological changes occurred in these secretory cells during the

year. The scarcity of secretory material, relative to the amounts present in cell Types 1 and 2, and its occurrence as small granules made it difficult to determine what proportion of cells showed secretory activity. It was concluded, however, that no major histological changes occurred in the other neurosecretory cell groups in the course of this study.

Discussion

Cell types

A few comments should be made regarding a comparison of the neurosecretory cell types of the crayfish with those described for other crustaceans by Enami (1951), Matsumoto (1954), and Carlisle and Passano (1953). Although these authors studied brachyurans and used fixatives other than those used by the present writer, their findings bear similarities with those reported here for the crayfish.

Table I

Counts of Type 1 and Type 2 neurosecretory cells containing secretory material in the brain and eyestalk of Orconectes virilis

Date	Number of animals	Cell type	Mean count and standard error
May 6	2	2	117±1
June 28	4	1	43±1
	4	2	125±8
July 23	4	1	46±2
	4	2	53±5
August 14	3	1	51±2
	3	2	66±4
August 31	4	1	38±2
	4	2	38±4
September 22	3	1	40±1
	3	2	38±6

Of the four neurosecretory cell types described above for the crayfish, it has been shown that the Type 1 and Type 2 cells are practically restricted in their distribution to the x-organ; only a few Type 1 cells are found in other neurosecretory cell groups. A study of Enami's figures reveals that in *Sesarma*, the giant beta neurosecretory cell is the only type found in the x-organ of that animal. Smaller beta neurosecretory cells are present as a small paired group in the supraoesophageal ganglion of *Sesarma*. Enami reports that the cytoplasma of the beta neurosecretory cells is fairly homogeneous and of compact appearance, showing but slight contraction upon fixation. It is apparent from other figures in Enami's paper that the Type 2 cells in the crayfish are similar to the giant beta cells in *Sesarma*.

No Type 2 neurosecretory cells were found in the brain of the crayfish. The crab, *Sesarma*, would appear to differ from the crayfish in that the crab possesses a paired group of beta neurosecretory cells in the supraoesophageal ganglion. In addition, Enami describes no cells in *Sesarma* which are comparable to the Type 1 cells of the crayfish.

The Type 3 cells of the crayfish are comparable to the alpha celis of *Sesarma*

in their distribution and in some of their cytological details. In both animals they are found in all neurosecretory cell groups except the x-organ. Both cell types are rich in cytoplasm and are characterized by the presence of vacuoles which are sharply delimited from the cytoplasm.

The Type 4 neurosecretory cells of the crayfish are similar to the gamma cells of *Sesarma*. They correspond in all features to the gamma cells. Small size, little cytoplasm relative to the size of the nucleus, and scarcity of secretory material are characteristic of these cells in both animals.

Of the four neurosecretory cell types described by Matsumoto (1954) for *Eriocheir japonicus*, he compares only his C cells, located in the ventral ganglion, with Enami's beta cells. However, Enami has shown that no beta neurosecretory cells occur in the ventral ganglion of *Sesarma*. Judging from the figures in their papers and the cell types observed in the crayfish, it appears possible that Matsumoto's C cells might be more comparable to Enami's alpha cells and to the crayfish Type 3 cells.

Carlisle and Passano (1953) found three types of neurosecretory cells in the x-organs of most species of crustaceans they examined. However, the number of cell types later was reduced to two (Carlisle, 1953). These authors showed that in the Natantia, the x-organ is divided into two portions, the pars ganglionaris which is located on the medulla terminalis and the pars distalis which is located elsewhere in the eyestalk. The Brachyura and the crayfish, in contrast to the Natantia, possess an undivided x-organ. Also, Carlisle and Passano found one neurosecretory cell type to be located in the pars ganglionaris x-organi and the other in the pars distalis x-organi. The cells of the pars ganglionaris x-organi are comparable to the giant beta neurosecretory cells of *Sesarma*, and Carlisle and Passano referred to them as the *x-organ neurosecretory cells*. It is evident that, since the Type 2 neurosecretory cells of the crayfish are comparable to the giant beta neurosecretory cells of *Sesarma*, they are also similar to the *x-organ neurosecretory cells* described in the Natantia by Carlisle and Passano.

There is a close parallelism in the arrangement of neurosecretory cell groups of the crayfish and the land crab, *Gecarcinus* (Bliss, Durand and Welsh, 1954). Furthermore, a comparison of Figure 8 of the present paper with Figure 11 in Enami's paper (1951) has already revealed that there is a remarkable similarity in the distribution of neurosecretory cell types in the crayfish and *Sesarma*. The parallelism in the distribution of neurosecretory cell types in the crayfish and *Sesarma* is particularly interesting when the physiological role of these cells is considered. This is discussed in the next section.

Secretory activity

When considering the increase in secretory activity that was observed in one type of neurosecretory cell in May, it should be remembered that the animals used in May had been kept in the laboratory for three to four months. There is evidence that crustaceans kept in the laboratory for long periods of time are different from those freshly collected. The molt-promoting effects of constant darkness on *Gecarcinus* (Bliss, 1954) are slowed down or delayed when freshly collected crabs are used. Animals long maintained in the laboratory respond quickly (Bliss, personal communication). *O. virilis*, kept in the laboratory, have been observed to

molt in fairly large numbers in May and the first part of June. Although no observations were made on the molting of *O. virilis* in the field in May and in early June, it seems reasonable to assume that the laboratory stock animals molt at an earlier date than animals in the field because of the higher temperatures and more regular food supply that probably exist under laboratory conditions. Therefore, data from the February and May animals used in this study may not be strictly comparable to data from crayfish that were freshly collected.

The results included in Table I raise a question concerning the physiological significance of the increased content of stainable material in the Type 2 neurosecretory cells of the x-organ just prior to molt. The idea that the sinus gland is a storage-release center for neurosecretory products (Bliss, 1951, 1953; Bliss, Durand and Welsh, 1954; Bliss and Welsh, 1952; Passano, 1951a, 1951b, 1952, 1953) implies that there is a mechanism whereby the rate of release of the substances can be controlled. Indeed, the well known reactions of certain crustaceans to background color are evidence that the release of certain neurosecretory products, *e.g.*, chromatophorotropins, is precisely regulated. Since a molt-inhibiting substance is produced in the x-organ and passed to the sinus gland for release into the blood stream, it is necessary to assume that at some time before the animal molts there is a decreased synthesis of this substance in the cells of the x-organ, a decreased release from the sinus gland, or both. It is assumed here that the release of the molt-inhibiting substance is decreased before molt.

It is known that the pars ganglionaris x-organi of the Natantia produces a molt-inhibiting hormone (Carlisle, 1954) and is comparable to a portion of the x-organ in the crayfish (this paper). Since the crayfish x-organ probably produces a molt-inhibiting hormone and since the only neurosecretory cells present in the pars ganglionaris x-organi of the Natantia are comparable to the Type 2 neurosecretory cells of the crayfish, it is conceivable that this neurosecretory cell type is the source of the molt-inhibiting hormone. If this is so, then the accumulation of stainable material found in the Type 2 neurosecretory cells just before molt can be considered evidence of more (1) precursor of the molt-inhibitor substance, (2) carrier substance, or (3) active material.

It is evident from the cell counts of the May animals that an assumed reduction in the rate of release must occur over a rather long period before molt. In adult crayfish large amounts of secretory material are present in these cells early in May, and signs of this increase are found in February in laboratory crayfish. Preliminary studies show that in immature crayfish possessing an intermolt period of approximately thirty-five days, increased amounts of secretory material are present in Type 2 neurosecretory cells of the x-organ at least five days before molt.

Fewer Type 2 neurosecretory cells contain secretory material after molt. This could result, if, after molt, there is a sudden release of stored material from the sinus gland and a rapid transfer of material from the cell bodies to the sinus gland for further release. Pyle (1943) found pronounced changes after molt in both the amount and staining qualities of the sinus gland material. He fixed eyestalks from *O. virilis* a few hours before molt and after the animals had completed molt. He found that there was a sharp reduction in the number of secretory granules present in the sinus glands after molt. Since the secretory masses he refers to in his photographs are identical in appearance with similar masses observed by the present

author in neurosecretory fiber endings in the sinus glands, it is possible that practically all of the material in a given axon ending is released after molt. In the crayfish this release takes place in a period of not more than a few hours (Pyle, 1943).

The accumulation of stainable material in the Type 2 neurosecretory cells of the x-organ prior to molt has been explained on the basis of a hypothetical witholding of molt-inhibiting hormone by the sinus gland and a continued synthesis of hormone or its precursor in Type 2 neurosecretory cell bodies of the x-organ. The secretory

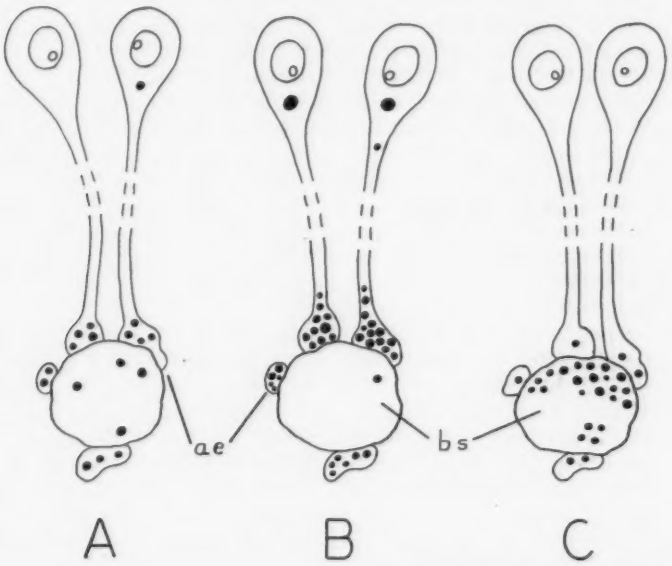


FIGURE 11. Hypothetical scheme for the secretory activity of Type 2 neurosecretory cells. A. During the intermolt period, a slow release of secretory material into the blood and synthesis of the material in Type 2 neurosecretory cell bodies continues. B. Shortly before molt, release of neurosecretory material into the blood is decreased; synthesis of the material in the Type 2 neurosecretory cell bodies continues. Material thus accumulates in the axon endings and in the cell bodies. C. Immediately after molt, a sudden release of secretory material into the blood occurs; cell body secretory material is transferred quickly to the axon endings for release. ae, axon ending; bs, blood sinus.

activity of the Type 2 neurosecretory cells is summarized in Figure 11. This is in complete agreement with the existing hypothesis on the control of molt in crustaceans (Bliss, 1953; Bliss, Durand and Welsh, 1954; Bliss and Welsh, 1952; Passano, 1953). It is interesting that the only neurosecretory cells of the eyestalk that show histological changes correlated with molt are restricted to the x-organ, the only cell group so far proved to be effective in the prevention of molt (Passano, 1953). As to the functions of the other neurosecretory cell types in the crayfish, no information was obtained in this study.

Summary

1. There are four cytologically distinct types of neurosecretory cells in the eyestalk and brain of *Orconectes virilis*. Two of these neurosecretory cell types are restricted in their distribution to the x-organ. The other two cell types occur in all neurosecretory cell groups in the eyestalk and brain except the x-organ.

2. The distribution of neurosecretory cell types has been compared with that described by Enami (1951) for *Sesarma*.

3. The Type 2 neurosecretory cells are the only neurosecretory cells that undergo histologically demonstrable changes in secretory activity in relation to the molting cycle. It is suggested, therefore, that the Type 2 neurosecretory cells are the source of the molt-inhibiting hormone.

4. Arguments are presented in favor of the view that at some time before molt a decrease occurs in the rate of release of molt-inhibiting hormone from the axon endings of the Type 2 neurosecretory cells. This decrease seems to be correlated with a concurrent accumulation of stainable material observed in Type 2 neurosecretory cell bodies.

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THE PRESENCE AND SIGNIFICANCE OF RESPIRATORY METABOLISM IN STREAK-FORMING CHICK BLASTODERMS[1]

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The value of simple sugars, particularly glucose, in early chick development is well known (Needham and Nowinski, 1937; Spratt, 1949; Taylor and Schechtman, 1949; Fraser, 1954a). Needham (1931) and Romanoff and Romanoff (1949) list glucose as a free constituent of egg yolk, and recent investigation (Fraser, unpublished results) has shown that glucose is the only free monosaccharide in egg white dialysate detectable by chromatographic procedure. In 1938 Jacobson found that there was a marked glycolysis in involuting mesodermal cells at gastrulation. Such studies have illustrated the importance of carbohydrate metabolism in early chick embryogenesis.

While the importance of carbohydrate utilization is generally recognized, the manner in which it is metabolized has been disputed. Novikoff, Potter and LePage (1948) stand against the contention of Needham and Nowinski (1937) that there is a non-phosphorylating glycolytic scheme in young chick blastoderms. The former authors were able to detect assorted phosphorylated carbohydrate components in embryos of three to ten days incubation. Needham and Nowinski were unable to find an increase in oxygen consumption either in whole embryos or homogenate, when phosphorylated sugars were added.

Aside from the energetics involved at this level of glycolysis, terminal oxidation with molecular oxygen by cytochrome oxidase has been followed. Using manometric means, Potter and DuBois (1942) found the first evidence of this enzyme in the six-day embryo. Albaum and Worley (1942) were able to detect activity, as measured by oxygen uptake, in the embryo of four days. By soaking blastoderms in solutions containing dimethyl-p-phenylenediamine and alpha naphthol (nadi reagent), followed by visual inspection, Moog (1943) has been able to show that cytochrome oxidase is present even in head process stages. Sodium azide-treated embryos lost much of the respiratory activity seen in the experimental group.

Moog also found that this enzyme activity was expressed in a morphological pattern similar to that displayed by reducing enzymes (Spratt, 1951a), sensitivity to respiratory poisons (Hyman, 1927; Spratt, 1950b), anaerobiosis (Spratt, 1950a) and starvation (Spratt, 1951b; Fraser, 1954a). In general, these experiments have revealed that the node and fore-brain are regions of high metabolic activity. I (Fraser, 1954a) have been able to demonstrate that the node is very susceptible to degenerative changes on starvation, while the brain is relatively refractory to such treatment.

It is the purpose of the present paper to determine if there is indophenol oxidase

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(cytochrome oxidase according to Keilen and Hartree, 1938) activity in earlier, streak-forming stages of the chick, and if so, if there is some pattern in its distribution. Further, if certain cells do show a greater respiratory metabolism, another objective in mind is to investigate the possibility that it has some significance in the differentiation of these cells.

The results from the present work permit the following statements. Cytochrome oxidase is detectable in streak-forming chick blastoderms, particularly in the newly involuted mesoderm cells. There is no change in the activity of this enzyme in embryos on the nadi reagent following pretreatment with cytochrome *c* or albumen, but it increases appreciably in blastoderms starved for five hours in saline. There is a striking decrease in its activity, as measured by indophenol formation, in explants treated mildly with hydroquinone, a reductant presumably for cytochrome *c*. The augmented respiratory metabolism seen in newly formed mesoderm cells is related in some manner with differentiative ability at the trunk level of the chick blastoderm, since fragments of involuted mesoderm expressed ability to form mesoderm tissue, while potential mesoderm fragments (axial epiblast in broad- and intermediate-streak embryos) failed in this respect.

Experimental Procedures and Results

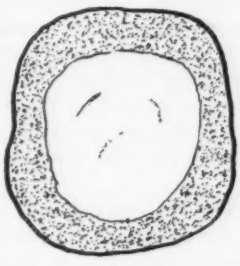
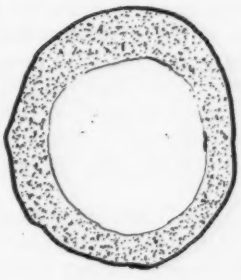
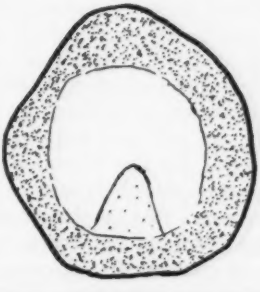
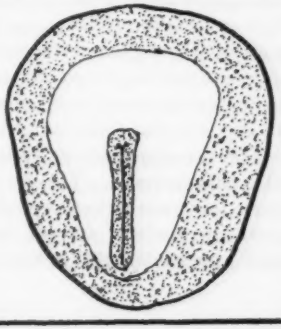
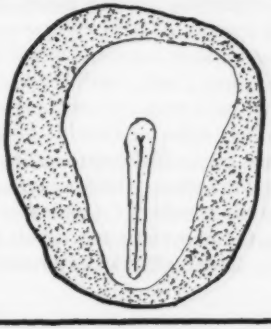
Newly laid fertile eggs were obtained from the poultry farm of the University of Tennessee. Most of the eggs used were from White Leghorn chickens, although a few were from Rhode Island Red stock. They were stored in the refrigerator at 18° C. upon receipt until the time (within five days) they were used in the experiments. The procedure for removing blastoderms from the yolk and for explanting them in culture has been given previously by Spratt (1947). Eggs were incubated in a forced draft incubator at 37.8° C., while explants were cultured at 37.6° C. The temperature in both incubators was maintained at a constant value by mercury type thermoregulators. Manipulation of the blastoderms was carried out under Ringer's solution. Specimens for histological inspection were fixed in Bouin's fluid and stained with Delafield's hematoxylin. Over six hundred blastoderms were used in the course of this investigation.

A. Aerobic enzyme pattern in early-streak blastoderms

Pre-, intermediate- and definitive-streak (DPS) embryos corresponding to stages 1, 2–3 and 4 of Hamburger and Hamilton (1951), respectively, were removed from the yolks under saline, and explanted onto freshly prepared nadi reagent, made in the following manner. Five ml. of 0.08 *M* dimethyl-p-phenylenediamine in chick Ringer's, 5 ml. of 0.08 *M* alpha naphthol in 20% ethyl alcohol-Ringer's, 1 ml. of bicarbonate buffer and 2 ml. of phosphate buffer were added to 27 ml. of Ringer's containing 300 mg. agar, after the saline-agar had been heated and then cooled to approximately 50° C. In every experiment outlined here and subsequently in which the nadi reagent was used, fresh preparations of dimethyl-p-phenylenediamine and alpha naphthol were made up immediately before use, because the former compound is oxidized rather rapidly by atmospheric oxygen. Preparation of the buffers used has been described previously (Fraser, 1954a). At this time, however, saturation of the bicarbonate buffer with CO_2 was achieved by passing the gas from a tank through a nozzle into the solution. The final pH of the 0.01 *M* nadi reagent

TABLE I

Pattern of cytochrome oxidase activity in early chick embryos explanted onto media containing dimethyl-p-phenylenediamine and alpha naphthol (nadi reagent) and nadi reagent with sodium azide

NO.	GENERALIZED RESULTS	
	NADI REAGENT (.01 M)	NADI REAGENT (.01 M) Na AZIDE (.005 M)
36		
34		
38		

medium was 7.1. The preparation was poured immediately into watch glasses held in petri dishes by moisture-saturated cotton rings. Gelation of the medium occurred within a few minutes. A control medium containing sodium azide at a final concentration of 0.005 *M* was made up in a similar manner.

The embryos were explanted onto the nadi reagent or nadi reagent-azide media and incubated for fifteen minutes at 37.6° C. At precisely this time they were removed from the media by pipette and examined under saline against a white background through a dissecting microscope.

The generalized observations are shown in Table I. Cells in the opaque area peripherad to the germ wall in all three stages tested showed a dark blue-purple coloration on the nadi reagent. No other pattern of coloration indicative of cellular respiration could be found in pre-streak embryos. A dark color was apparent, however, in the streak-forming region of the intermediate-streak blastoderm and along the streak and node area in the DPS explants. Other pellucid area tissues were very faintly stained. On the azide-bearing medium, the yolk-laden cells in the area opaca retained, in large part, a deep coloration, while streak tissues were essentially colorless. In fact, it was extremely difficult to see the developing or full streak against the white background in embryos removed from the medium containing the azide.

The logical interpretation to be made from the above results is that the coloration expressed in the vitelline cells surrounding the embryo is due mainly to a non-enzymatic mechanism. Since interest was directed toward a localization of activity in the embryo proper, this issue was not pressed, although it may be conjectured that the amount of tissue and yolk in this region may be sufficient to soak up enough indophenol from the surrounding fluid to give this appearance. Other interpretations may be advanced, but failure of the azide to prevent coloration must mean that known oxidative enzymes are not involved. Furthermore, the marked depression in indophenol formation in streak tissues on the azide medium must indicate that this coloration is mediated by enzyme action. There is good evidence that azide inhibits the action of indophenol oxidase (cytochrome oxidase) which is directly responsible for the formation of the bluish-purple indophenol (Keilin, 1936) as well as transphosphorylation and ATPase activity (Meyerhoff, 1945).

To insure that the darker color in the region of the forming and full streak was not due simply to the presence of more cells compacted at this region, fragments of tissue of comparable thickness were removed from streak epiblast, mesoderm and hypoblast for inspection. For comparison, fragments of non-axial epiblast were also examined. These pieces were placed side by side on a microscope slide in a small amount of fluid and covered with a cover slip. The stained tissues prepared in this manner were examined for intracellular indophenol deposition.

Figures 2 and 3 will reveal that enzymatic activity is greater in involuted mesodermal cells than in overlying epiblast (potential mesoderm in intermediate-streak embryos) cells. Attention is drawn to the fact that indophenol is produced at the surface of small droplets in the cells. The cytoplasm of the cells is relatively free from coloration. This is typical of all cells observed. It may also be seen that while the number of droplets is essentially the same in both epiblast and mesoderm cells, the enzymatic activity is greater on the surface of those in newly involuted cells. These globules are readily stained with Sudan III, indicative of a lipid con-

tent. These photographs are of living cells removed from a streak-forming blastoderm. In obtaining the photographs care was taken to make sure that identical conditions, such as illumination, exposure time, time of processing, etc., were maintained. The similar appearance in the photographs of a defect in the lens of the photographic equipment will attest this. Such inspection revealed that as far as indophenol oxidase activity is concerned, streak hypoblast and all epiblast cells tested were the same. It is clear that the darker coloration of the region of the forming streak or in the node and full streak of the older blastoderms is due solely to a greater respiratory activity in newly involuted mesoderm cells. This observation is in conformity with that of glycogen utilization by invaginating mesoderm made by Jacobson (1938).

B. *Modification of cytochrome oxidase activity by pretreatment*

We are dealing here with an enzyme which has as its substrate the nadi reagent under experimental conditions and presumably cytochrome *c* in normal cellular respiration. Therefore, on theoretical grounds at least, it should be possible to modify the reaction between the enzyme and the nadi reagent by the addition of the normal substrate. If living cells behave as does mammalian heart muscle extract, according to the observations of Keilen and Hartree (1938), we should expect an increase in oxidation of the diamine on addition of cytochrome. At the same time, a depletion of readily metabolizable food reserves in the cell, resulting in a depressed enzyme activity, could also conceivably lead to greater nadi oxidation, and hence augmented coloration. These ideas were followed by the following experimentation. Four dozen eggs were supported on their sides in a tray and left in the refrigerator at 18° C. overnight, so that the position of the blastoderm would be known. On the following day 0.2 ml. of 1.4×10^{-4} *M* cytochrome *c* was injected into the yolk sacs of two dozen eggs, while a similar quantity of 2% sodium succinate was injected into the other two dozen. Based on previous measurements of frozen eggs a needle of sufficient length was chosen so that the injected materials would be placed about one quarter inch from the blastoderm. The needle was inserted vertically from the lower side of the egg to avoid possible injury to the blastoderm. Both preparations that were injected were sterilized by filtration. The concentration of the cytochrome *c*, prepared according to Umbreit *et al.* (1949), was established by use of the Beckman spectrophotometer. The eggs thus treated were incubated for ten hours after which they were explanted onto the Nadi reagent in the manner described above.

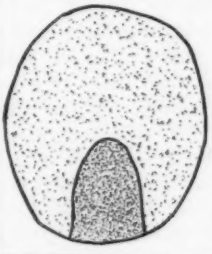
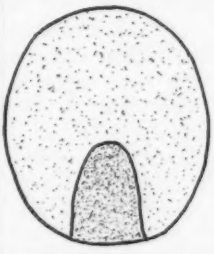
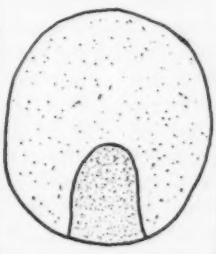
The results were rather disappointing in that there was no difference in enzymatic activity in either of these groups of embryos as compared to normal, non-injected controls. It became clear that the question as to whether the negative results were due to the inactivity of the materials on the blastoderms or to the failure of the materials to reach the embryos could not be resolved by this procedure, so it was abandoned.

Next, early streak blastoderms were removed from the yolk in the usual manner after ten hours of incubation, and incubated in various liquid preparations. These included: (1) saline, (2) albumen, (3) albumen-cytochrome *c*, (4) albumen-cytochrome *c*-hydroquinone and (5) albumen-hydroquinone. The final concentration of cytochrome *c* was $1.4 \times 10^{-5}M$, that of hydroquinone $10^{-3}M$. The albumen con-

centration was the same as previously employed in agar gels. Pretreatment time for those in the first three media indicated was five hours. It has been shown (Spratt, 1951b; Fraser, 1954a) that permanent damage occurs in embryos explanted on non-nutrient media for intervals longer than this. Blastoderms were pretreated in the media containing hydroquinone for one hour, since beyond this time cell dispersal began. Twenty-four embryos were placed in each medium, incubated for the period indicated above, removed, washed thoroughly in saline and immediately explanted onto a nadi reagent-bearing agar medium. On this they were again incubated for fifteen minutes after which they were removed, placed in Ringer's and inspected. Embryos of the same age were removed directly from eggs and treated on the nadi reagent for the same length of time for comparison.

TABLE II

Relative expression of cytochrome oxidase activity in streak-forming chick blastoderms pretreated with materials indicated

	GENERALIZED RESULTS		
PRETREATMENT MEDIUM	SALINE	ALBUMEN ALBUMEN–CYTOCHROME C CONTROL	ALB–CYT.C–HYDROQUINONE ALBUMEN–HYDROQUINONE
			
NUMBER	24	60	48

The results are shown in generalized form in Table II. To avoid confusion, the three groups of media producing different results will be treated separately.

(1) *Albumen, albumen-cytochrome c, control* (*no previous treatment*): the sixty blastoderms in this group were all similar to those described previously with respect to indophenol oxidase pattern and intensity of color. It appears obvious that neither the albumen nor the cytochrome *c* had any affect on the enzyme activity when presented to the embryos in this manner.

(2) *Saline* (*non-nutrient*): Blastoderms incubated in this fluid stained most intensely by nadi reagent. All embryonic tissues were slightly darker than those on media listed above, but streak tissue was considerably more colored. A comparison of cellular details in fragments of tissues from streak epiblast, mesoderm and hypoblast between these and albumen-treated embryos revealed that coloration was darker in all three germ layers in prestarved embryos, with newly involuted mesoderm again showing the greatest indophenol deposition (Fig. 4).

(3) *Albumen-cytochrome c-hydroquinone, albumen-hydroquinone:* After pretreatment in these media, blastoderms showed a striking decrease in indophenol coloration when incubated on the nadi reagent. All tissues seemed to be stained somewhat less, but again the streak-forming tissues seemed most influenced by pretreatment. Although not nearly as faintly colored as those on an azide medium, these tissues nevertheless were considerably lighter in appearance than in control blastoderms.

In all cells observed, the blue color was localized on the surface of intracellular globules, even in starved embryos. It is interesting to note that cells showing the greatest enzyme activity are those of starving blastoderm mesoderm, and that this activity is on the surface of lipid material. The significance of this and of other observations made at this time will be discussed more fully later. It will suffice to point out here that the less intense color on the droplets in cells of hydroquinone-treated animals represents a decrease in dimethyl-p-phenylenediamine-alpha naphthol oxidation. This is what one would expect if one assumed that the hydroquinone acts specifically as a reductant (as has been shown by Krahl and co-workers, 1941, in sea urchin eggs) for intracellular cytochrome *c* and not indophenol, and provided that the cells were not killed by such treatment.

Considering the first assumption, it was determined that hydroquinone, in the concentration used in the experiment, and even in much greater concentration, could neither prevent the formation of indophenol from fresh nadi reagent, nor could it reduce indophenol to the leuco form *in vitro*. Secondly, other blastoderms treated with hydroquinone as indicated were subsequently washed thoroughly and explanted onto an albumen-agar medium. These were then incubated for twenty-four hours. Although development did not proceed as in normal explants, there was some slight morphogenesis, and tissues did not have an opaque appearance characteristic of death of the cells.

C. *Non-autonomy of the increase in cytochrome oxidase activity*

The question arose as to whether the increase in enzymatic activity seen in involuted mesoderm was a function of time or of location of tissue. Preceding statements have indicated that after a pretreatment interval of five hours, there was less indophenol localized in epiblast cells than in mesodermal cells. But coincident with change in time there has been some involution during pretreatment.

Small fragments of tissue taken from streak epiblast and streak mesoderm were removed from streak-forming blastoderms and cultured under albumen (prepared as previously outlined) for intervals from five to ten hours. These were then washed in saline and explanted onto the nadi reagent medium for fifteen minutes and inspected. Similar fragments removed from blastoderms of the same age, but removed directly from eggs, were stained as controls.

The cultured streak epiblast tissue had the same blue indophenol coloration as the controls. Streak mesoderm cells from both groups also looked identical, although darker in appearance than epiblast cells.

By preventing involution in this manner in epiblast tissue (prospective mesoderm), cultured for a sufficient period of time for this basic morphogenetic phenomenon to have occurred, it was thus possible to show that increase in indophenol oxidase activity is not autonomous in this tissue. It seems clear that the gain in

respiratory activity is either due to movement of cells through the streak in gastrulation or to the influence of surrounding cells in a new mesodermal location. The following experiments are directed toward this question.

D. *Significance of increased respiratory activity of cells in histogenesis*

If an increase in enzyme activity in cells is due simply to placement of the cells, it should conceivably occur in streak epiblast cells implanted in a mesodermal location in early chick embryos. Eighteen fragments of such tissue were implanted through small tears in the hypoblast into positions indicated in Figure 1. Fragments were removed from stage 2 (Hamburger and Hamilton, 1951) embryo streak epiblast, while hosts were of stages 2 and 4. Blastoderms with implanted tissue were then incubated for six hours on a regular albumen-agar medium, after which

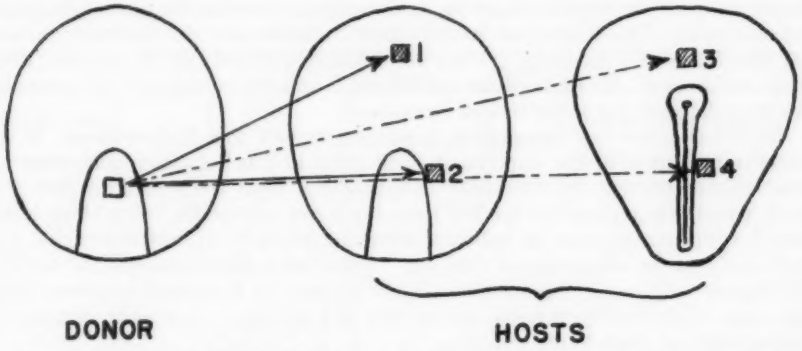


FIGURE 1. Illustration of sites for implanting fragments of streak epiblast into mesodermal locations of intermediate-streak and DPS blastoderms.

they were removed and explanted onto the nadi medium and reincubated for fifteen minutes. The implants, when found, were then removed under saline and mounted on microscope slides along with nadi-stained fragments of epiblast removed directly from streak-forming embryos for comparison. About one-third of the implants were extruded during incubation on albumen and were hence lost.

Fragments placed at mesodermal positions 2 and 4 in Figure 1 looked identical to control pieces. There had been no increase in enzyme activity in cells placed in these positions. From this and the foregoing observation, we might conclude that the increase in cytochrome oxidase activity in newly involuted cells is neither a direct function of time nor location, but is tied in with the morphogenic movement of the cells through the streak. This at least seems true for cells at the level of the streak in the early chick blastoderm.

It was rather surprising to see that implants into mesoderm in the prospective head region (positions 1 and 3 in Figure 1) were considerably darker than the controls. On the cell level, there was perceptibly more indophenol on the globules in cells of the implants than of the control fragments. At this region of the embryo, it appears that the development of intracellular catalytic activity is related to location of the cells.

These experiments were then followed by others to test the significance of this increase in observed enzymatic activity with respect to differentiative ability of the tissues involved. Fragments of streak epiblast were excised from streak-forming embryos and marked very lightly with diluted India ink under saline. They were then placed in saline and larger particles of carbon were removed with a steel needle. After a final washing, the pieces of tissue were implanted into mesodermal sites indicated in Figure 1, in both streak-forming and DPS hosts. Implantation occurred through small tears made in the hypoblast at the desired regions. In a similar manner, small pieces of newly involuted mesoderm from the same embryos were implanted in the same regions with the exception of the head region in early streak embryos. The host blastoderms were then cultured on albumen-agar for twenty-four hours at 36.7° C., fixed with Bouin's fluid and prepared for paraffin impregnation. The serial sections were made 12 microns in thickness. Eighteen implants were made into each site indicated.

TABLE III

Summary of results from implanting fragments of streak epiblast of streak-forming (SF) chick embryos into mesodermal sites of host embryos.

Type of fragment	Site of implantation	Fragments recovered*	Results
Epiblast	Head SF	12	Head mesoderm and pharynx
Epiblast	Head DPS	9	Head mesodern and pharynx
Epiblast	Flank SF	15	Isolated ball degenerative cells
Epiblast	Flank DPS	13	Isolated ball degenerative cells
Streak meso.	Head DPS	12	Head mesoderm and pharynx
Streak meso.	Flank SF	10	Ball of living cells Flank mesoderm
Streak meso.	Flank DPS	10	Ball of living cells Flank mesoderm

* Number of blastoderms showing some evidence that tissue had been implanted.

The results are given in Table III. It will become immediately evident that there is a good correlation between increased cytochrome oxidase activity and differentiative ability. Fragments of epiblast placed in prospective head mesoderm not only show an increase in metabolic (oxidative) activity but also display an expanded capacity for differentiation. Implants in flank regions failed to show any increase in enzymatic activity coincident with a failure to produce mesodermal structures. It is also apparent that involuted mesoderm has the capacity to form both mesoderm and endoderm in the head region as well as flank mesenchyme. There are limitations to this ability at the trunk level, however. Figures 5–10 are photographs illustrating the results obtained. Engulfed particles located in head mesoderm and pharynx are taken as evidence that these cells had differentiated from implanted tissue.

DISCUSSION

The work of Moog (1943) has established that cytochrome oxidase is present in chick embryos in stages as early as those possessing a head process. In view of the fundamental importance of this enzyme in many diverse organisms, there

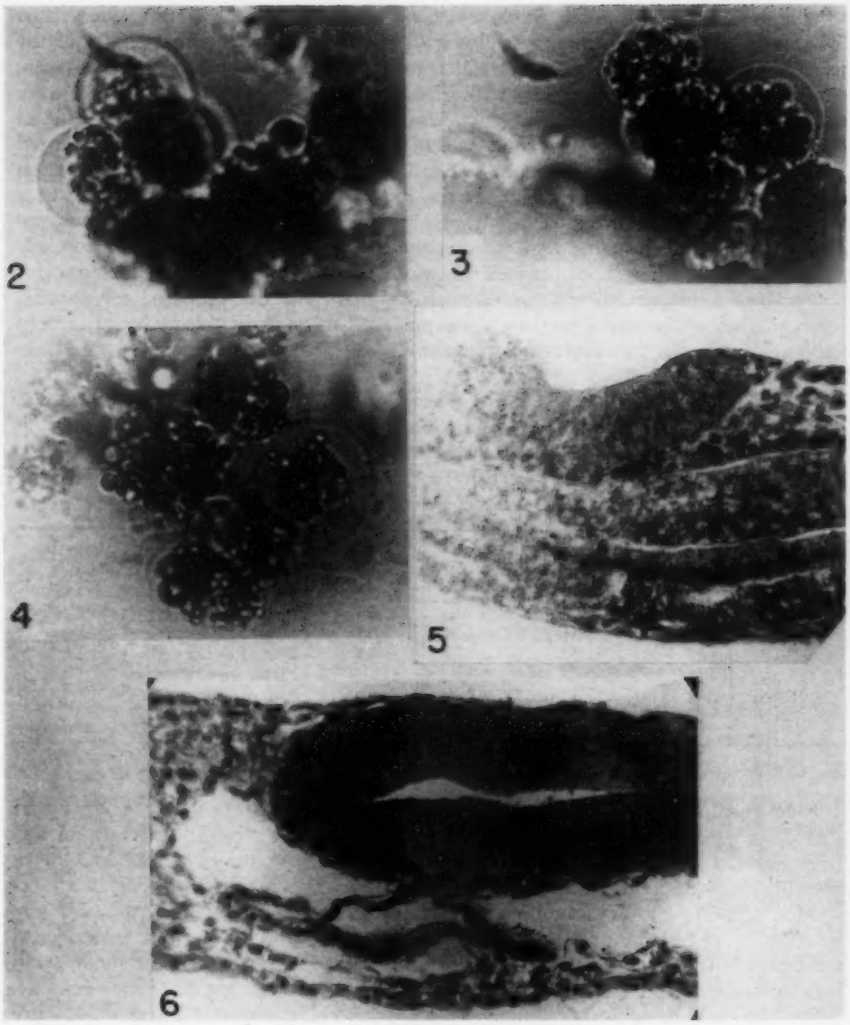


FIGURE 2. Living nadi reagent-treated cells from streak epiblast of an intermediate-streak blastoderm. Note that indophenol is deposited on the surface of intracellular droplets. × 960.

FIGURE 3. Photomicrograph of living cells stained on the nadi reagent. These cells are from newly involuted mesoderm of an intermediate-streak embryo. × 960.

FIGURE 4. Stained streak mesoderm cells from a pre-starved intermediate-streak blastoderm. × 960.

FIGURE 5. Carbon-marked cells in pharyngeal endoderm of a stage 2 explant after twenty-four hours of subcultivation. The brain at this level has not rolled completely into a tube. × 120.

FIGURE 6. Carbon engulfed by mesoderm and pharynx of a DPS explant after one day of subcultivation. ×120.

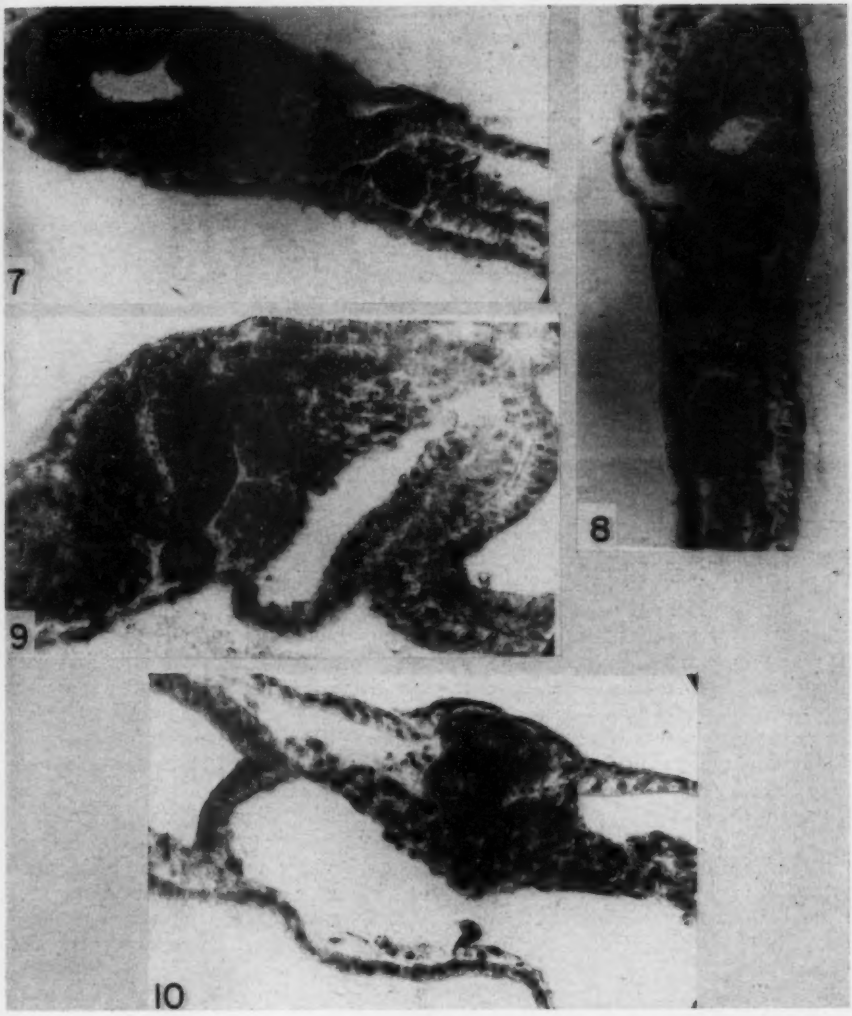


FIGURE 7. Remnant of an epiblast implant grafted into flank mesoderm of a DPS host followed by twenty-four hours of incubation. ×120.

FIGURE 8. Photomicrograph of an isolated ball of degenerative epiblast cells in flank mesoderm of a stage 2 blastoderm host after one day of subcultivation. × 120.

FIGURE 9. Isolated mass of viable cells in mesoderm initially implanted in the flank region of a DPS host. Photograph taken twenty-four hours postoperatively. Donor cells were from newly involuted mesoderm of a stage 2 blastoderm. × 120.

FIGURE 10. Photomicrograph of carbon-marked flank mesoderm cells in a stage 2 host following one day of subcultivation. Donor tissue was newly involuted mesoderm from a stage 2 embryo. × 120.

is no reason to believe that it may not be present in even earlier chick blastoderms. It has been detected by Krahl and co-workers (1941) in pre- and post-fertilized Arbacia eggs. Regardless of the type of food being utilized, as long as aerobic oxidation occurs it seems likely that this terminal enzyme will be involved. It is common knowledge that the chick embryo uses oxygen continuously following laying of the egg. The results of the present investigation have shown that cytochrome oxidase is indeed present in very early embryos, detectable by the nadi reagent under the conditions utilized even in streak-forming stages. Before this time it is probable that this enzyme is present but is not specifically outstandingly active at any localized region.

The specific significance of the observed rise in activity of cytochrome oxidase in involuting cells can only be conjectured. Certainly it is associated in some manner with the differentiative process, since it is accompanied by an expansion of possible fates in cells in which it occurs. It may well be that the increased energy liberated in these cells at this time may be directed toward anabolic processes. That differentiation is accompanied by aerobic oxidation has very recently been pointed out by Warburg (1956). The correlation between oxidative activity and histogenesis appears of prime significance.

Previously (Waddington, 1932; Fraser, 1954b) it has been shown that an interchange of cells between head endoderm (pharynx) and head mesenchyme is much in evidence in early chick embryos. It may well be that tissues in this region are more influenced in their differentiation by cells about them than they are at lower (trunk) levels. No evidence has been found in the present investigation to support the observations of Waddington and Taylor (1937) that epiblast tissue implanted at lower regions of the chick blastoderms would form mesodermal structures.

Mention should be made of the results obtained in experiments dealing with nadi oxidation in blastoderms pretreated with various materials. If we assume that there is competition between the nadi reagent and cytochrome *c* for the enzyme cytochrome oxidase, then certain interpretations of the results can fruitfully be made. This assumption is in sharp contrast to the ideas of Keilen and Hartree (1938), but see below. It is well known that both of these materials are oxidized by this enzyme in the presence of molecular oxygen to indophenol and oxidized cytochrome *c*, respectively. If we accept the assumption of a competition of substrates, the increase in nadi oxidation in starved embryos could mean that normal oxidation through cytochrome *c* is curtailed, presumably due to exhaustion of utilizable carbohydrate reserves (free hexoses). Dimethyl-p-phylenediamine-alpha naphthol would therefore be oxidized more readily, leading to the more pronounced coloration observed.

Indophenol intensity was the same in embryos pretreated with albumen-cytochrome *c* as in controls. At the same time, embryos incubated in albumen-cytochrome *c*-hydroquinone and albumen-hydroquinone were perceptibly less colored. If cytochrome *c* could enter the cells, we should expect a decrease in nadi oxidation. The fact that hydroquinone in the absence of cytochrome *c* produced the same result as with it suggests that the cytochrome is not gaining entrance to the cells. Krahl *et al.* (1941) and Keilen and Hartree (1938) using preparations of Arbacia eggs and mammalian heart muscle, respectively, have shown that there is an increase in cytochrome oxidase activity proportional to the amount of cytochrome *c* added. The size of the cytochrome (molecular weight of 13,000 according to

Potter, 1950) should not be a serious detriment to cell entry, since larger compounds are suspected of entering cells. Nevertheless, the results would indicate that it did not enter the cells. It is obvious, however, that the hydroquinone had. In view of the fact that this material will not reduce indophenol, but has been shown to reduce cytochrome *c* (Krahl *et al.*, 1941) one is led to the conclusion that the hydroquinone selectively and persistently reduces the normally present intracellular cytochrome *c*, and hence the affinity for the oxidase with the nadi reagent is reduced.

There is, however, an alternative explanation which is more in keeping with the conclusions of Keilen and Hartree (1938). These workers found that the catalytic action of cytochrome oxidase on p-phenylenediamine was greatly enhanced by the addition of cytochrome *c*, and that this aromatic amine was oxidized much more readily by the enzyme than were other compounds, including hydroquinone. They state further that the rate of catalytic hydroquinone oxidation may be increased 30- to 40-fold on the addition of cytochrome *c* (10^{-5} to 10^{-4} *M*) to the preparation. Thus, rather than there being a competition between cytochrome *c* and hydroquinone for the enzyme, in heart muscle preparations at least, there is a dependency on the presence of the cytochrome for the catalytic oxidation of hydroquinone. Assuming this and again considering only the hydroquinone as entering the cell, it must be that intracellularly the hydroquinone has more affinity for the enzyme than has the nadi reagent. This is, of course, somewhat at variance with the English workers' observations. It may be that the difference in results lies in the materials and methods used. Intracellularly, structure may provide results differing from those obtained *in vitro*. At any rate, this interpretation is also in keeping with observations made.

Finally, consideration should be given to the perplexing problem of starvation of the chick embryos whose cells are amply supplied with high energy food material. Reference has already been made to the fact that chick blastoderms soon die when explanted on non-nutrient media. Spratt (1951b) has shown that recovery is possible in embryos starved for six hours on a saline-agar medium, when they are returned to an albumen substrate. I have found (Fraser, 1954a) that certain degenerative features are obvious in explants starved for ten hours. In checking recently, I have found that in embryos starved for ten hours, there are still many intracellular Sudan III-stainable globules. It thus becomes evident that lipids are not utilized, at least to any appreciable degree, by early chick blastoderms. This conclusion had been drawn previously by Needham (1931). This author, using R.Q. determinations as a basis, stated that during chick embryogeny carbohydrates are utilized for the first seven days of incubation (R.Q. = 1), proteins are used next and finally lipids are used only near the time of hatching.

Summary

1. Cytochrome oxidase has been detected in chick blastoderms as early as the intermediate-streak stage, by use of the explanting procedure on an agar medium containing dimethyl-p-phenylenediamine-alpha naphthol (nadi reagent). Intracellular indophenol deposition was localized on the surface of lipid droplets, particularly in newly involuted mesodermal cells. Enzymatic activity was negligible in embryos explanted on a similar medium containing sodium azide.

2. Nadi oxidation was augmented, notably in streak mesoderm of early explants after such blastoderms had been starved in saline for a period of five hours. Embryos pretreated in albumen-saline, or albumen-saline-cytochrome *c* for a similar interval showed no increase or decrease in intracellular enzymatic activity as compared to controls, when they were subsequently explanted onto the nadi-bearing medium. However, diamine oxidation in blastoderms treated in solutions containing albumen-cytochrome *c*-hydroquinone and albumen-hydroquinone was perceptibly decreased.

3. The development of the ability to oxidize the nadi reagent was not autonomous in fragments of streak epiblast (prospective mesoderm), but required normal involution at gastrulation. This was shown by pieces of this tissue implanted into trunk-level mesoderm. When implanted in a future head mesoderm location, however, such fragments did reveal an increase in enzymatic activity. When incubated in albumen-saline for intervals of time up to ten hours, small pieces of epiblast did not show an increase in nadi oxidation.

4. These results were correlated with the ability of the tissue fragments to form mesodermal and endodermal structures. Implants of epiblast placed in prospective head mesoderm of streak-forming and definitive primitive streak hosts were incorporated into head mesenchyme and pharyngeal tissue. Similar tissue when placed with other mesoderm at trunk levels failed to differentiate into mesenchyme. Newly involuted mesoderm from streak-forming blastoderms had the same fate as did epiblast fragments, when implanted in a future head mesoderm location. At the trunk level this tissue became integrated into mesoderm cells about it or formed semi-isolated balls of living tissue.

5. The significance of the observations, with respect to nutritional requirements of early chick blastoderms and the relationship between oxygen utilization and differentiation, is discussed briefly.

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THE LOCATION OF CONTACT CHEMORECEPTORS SENSITIVE TO SUCROSE SOLUTIONS IN ADULT TRICHOPTERA[1]

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Descriptions of the mouth-parts and feeding of adult Trichoptera in recent American text books and other general works on entomology are not consistent. Folsom and Wardle (1934, pp. 20, 39), Frost (1942, p. 89), Comstock, (1950, p. 555), Metcalf, Flint and Metcalf (1951, p. 229) and Ross (1948, p. 367) described the mouth-parts as "vestigial," "rudimentary," "subatrophied," or "greatly reduced." These views carried as a corollary the belief that the adults take little or no nourishment, and Brues (1946, p. 44) listed caddis-flies with the "aphagia": insects "which do not feed at all after maturity." Borror and DeLong (1955, p. 437), however, described the mouth-parts as "chewing type, with the palpi well developed but with the mandibles much reduced," and stated that "the adults feed principally on liquid foods." Swain (1948, p. 79) also stated that adult caddis-flies take liquid food, but termed the mouth-parts a "short, uncoiled proboscis."

A review of earlier accounts reveals a similar lack of agreement. Réaumur (1737, pp. 175–176) wrote that the mouth-parts of Trichoptera are for sucking and lapping, like those of Diptera. Kirby and Spence (1826, p. 464, Pl. VII, Fig. 1), on the other hand, regarded the mouth-parts as modified mandibulate. Burmeister (1832, pp. 68; 377–378) stated that the mouth-parts are intermediate between the mandibulate and haustellate types, comparing them with those of bees. Lucas (1893) made a detailed study of the mouth-parts of *Anabolia furcata* (= *laevis*). He found the mandibles to be atrophied, the labrum and maxillae reduced, and the labium developed into a sucking organ, the haustellum. Ulmer (1904) and Cummings (1913, 1914) reported a well developed haustellum to be present in every family of Trichoptera. These facts are reported in the special works on Trichoptera by Betten (1934, pp. 19–22), Ross (1944, p. 4) and Mosely and Kimmins (1953, pp. 10–11), and in the text books of Packard (1898, pp. 74–75), Weber (1933, pp. 66–68; 1954, pp. 297–298), and Imms (1948, pp. 19, 411–412).

Réaumur (1737, pp. 175–176) stated that adult Trichoptera take liquid foods, and Burmeister (1832, pp. 377–378) reaffirmed this, reporting that he found them feeding on nectar of flowers. Lucas (1893) reported finding tiny particles like pollen in the folds of the haustellum, and he therefore believed that they feed on nectar. There were other workers who made observations, often quite casual, that confirmed or contradicted these ideas. These are reviewed in the papers of Siltala (1907) and Döhler (1914). Siltala (1907) gave adult *Phryganea striata* and *Limnephilus rhombicus* only water for three or four days, then placed a flowering

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branch of *Spirea* near them. They flew to the flowers and fed on the nectar, thus confirming the reports of feeding by adults.

Döhler (1914) made many observations that leave little doubt that adult Trichoptera feed. He fed them on sucrose solution to which litmus was added and followed the changes in acidity in the gut as an indication of digestion. He fed ferric lactate in water and demonstrated the absorption of iron by the gut-wall. When he gave only water to 23 individuals of *Limnephilus flavicornis*, they survived for 19–40 days. When he gave sugar-water to 19 others, they lived 45–105 days. He observed feeding closely by seizing the wings of the insects and holding them as he brought drops of sugar-water to their mouth-parts. He found, in *L. flavicornis*, such greedy acceptance of the food that some individuals ruptured the intestine through over-feeding. All these laboratory observations led Döhler to conclude that adult Trichoptera feed in nature, and he supported this by reference to Siltala's observations and those of earlier workers who reported finding caddis-flies on flowers or at sweet baits used to lure moths.

Lucas, Siltala and Döhler, thus, seem to have shown convincingly that the mouth-parts of some larger adult Trichoptera are functional. The labrum is reduced, the mandibles rudimentary, the maxillae modified, the maxillary and labial palpi well developed, and the hypopharynx or labium or both developed into an extensible haustellum. These species of Trichoptera, at least, probably feed in nature on nectar and other sweet substances.

The present paper reports experiments on four species of Trichoptera from two families, and observations on two other species from two more families. The purpose of the experiments was to discover the location of the contact chemoreceptors mediating feeding responses when stimulated with sucrose solution. The results further support the belief that the mouth-parts of adult Trichoptera are functional and that the insects feed in the adult stage.

Materials and Methods

The following species of Trichoptera were studied experimentally. Identifications were made by the authors with the aid of works of Betten (1934), Milne (1934–36) and Ross (1944). Dr. H. H. Ross kindly checked the identifications, and we wish to thank him for this.

Family: Phryganeidae
Banksiola smithi—2 males, 4 females
Ptilostomis ocellifera—1 male, 6 females
Phryganea sayi—4 males, 8 females
Family: Limnephilidae
Platycentropus radiatus—8 males, 9 females

All the experimental subjects were captured when they came to lights at night. They were lightly anaesthetized with ether and mounted alive by fastening the dorsal side of the thorax and the wings to a wax block on the end of a glass rod (Fig. 1). These are relatively large (*B. smithi* about 15 mm. long; the others 20–25 mm. long), and when they were thus mounted could easily be observed. Longevity was

good if the animals were fed and watered daily; even with legs and other parts removed they lived for up to 26 days.

The contact chemoreceptors were located by the methods described in detail in Frings and Frings (1949). The animals were tested daily after night fall, for they responded more actively at night, even with the necessary artificial illumination, than in day light, as Döhler (1914) also noted. Before each daily series of tests, the animals were given water to satiety. It was essential that water-satiety be maintained in the subjects, because the tests involved discrimination between water and water with sucrose added. When the animals had taken all the water they would take, water was brought to the locus being tested on an artist's brush or glass mi-

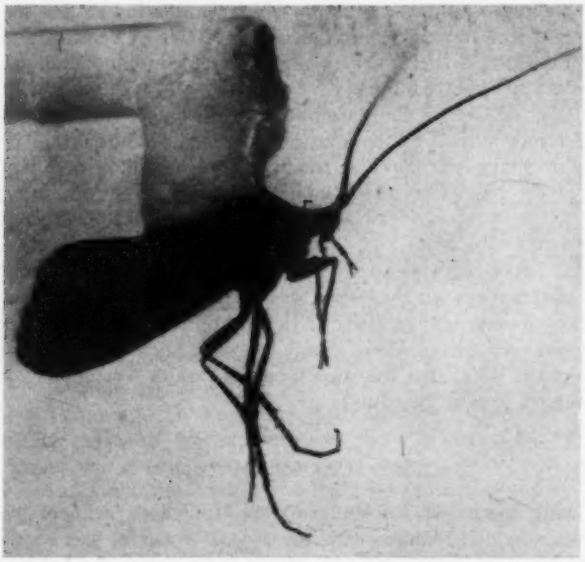

FIGURE 1. A caddis-fly (*Platycentropus radiatus*) mounted alive on a paraffin block on the end of a glass rod (3×).

croneedle and the reaction noted under a binocular dissecting microscope. This was followed by a similar trial with 1 *M* sucrose solution, and the reaction again noted. These presentations were repeated a sufficient number of times to be sure that the insect responded similarly or differently to the two stimuli. Such a series of trials constituted one test. There were variable numbers of tests carried out at each testing period. At the end of each daily testing period, the insects were fed to satiety on the sucrose solution. They imbibed heavily, but did not damage themselves, as Döhler reported for the animals he tested.

When preliminary experiments had revealed possible loci of contact chemoreceptors, the structures were removed and the animals retested similarly. Operations were performed under light anaesthesia with paired controls anaesthetized

and sham operated. For microscopic examination of possible end-organs on the experimentally determined loci, the structures were removed in 70% ethyl alcohol, transferred to 95% and thence to Diaphane on micro slides.

The following species were observed unmounted:

Family: Leptoceridae
Oecetis cinerascens
Family: Hydroptilidae
Orthotrichia americana

Attempts to mount and test about 30 individuals of the first-named were made, but these did not survive more than one day. Both of these species were very common and, as described by Döhler, highly attracted to sugar-water. They came onto the laboratory table and could be given water and sucrose solutions while they scurried about. By carefully controlling the placement of droplets near them, it was possible to test them and to observe feeding. *O. cinerascens* is large enough (about 12 mm. long) to be observed with the naked eye. *O. americana,* like all Hydroptilidae, is quite small (about 3 mm. long). It was necessary, therefore, to observe it with a dissecting microscope. Luckily the insects came right onto the brushes and needles used in testing the larger forms, and they were thus easily observed.

Results

With *B. smithi* only gross localization tests were made, using brushes with water and 1 *M* sucrose solution applied to various organs of the intact, mounted animals. The palpal tips proved to be quite sensitive: touching them with sugar-water brought about extension of the haustellum. The tarsi, likewise, had contact chemoreceptors: touching them with a brush bearing sugar-water induced eager reaching with the palpi toward the brush. With water alone on a brush, in each case, withdrawal or neutral reactions were elicited. Touching the antennae with water or sugar-water resulted in withdrawal of the antennae, indicating that these lack contact chemoreceptors sensitive to sucrose.

With *Pt. ocellifera, P. sayi* and *P. radiatus* more detailed experiments were carried out: with *Pt. ocellifera* about 100 tests were made over a period of 10–26 days; with *P. sayi,* about 200 tests over 16 days; with *P. radiatus* about 300 sets of tests over 16 days. These three species reacted almost exactly alike, and the results are thus given together. Figure 2 is a photograph of the head and mouth-parts of *P. radiatus* to show the well developed haustellum and palpi.

The first series of experiments was designed to give the general locations of the contact chemoreceptors. Using brushes in paired trials with water and 1 *M* sucrose solution, no evidence of discrimination was found when the antennae were tested. If the insects were "thirsty," however, the antennae were quite sensitive to water vapor. If a brush bearing water was brought near to but not in contact with the antennae, the insects almost immediately began to reach excitedly with the palpi. Once sated with water, however, this ceased, and the only reaction to con-

tact with a brush moistened with water or sugar-water was withdrawal of the antennae. The conclusion that the antennae lack contact chemoreceptors, however, must be stated cautiously, for recent experiments with some Lepidoptera (Frings and Frings, 1956) show that reactions mediated by the antennae may depend upon presence or absence of contact chemoreceptors on other parts of the body.

Contact of the tarsi of water-sated individuals with sugar-water elicited reaching with the maxillary and labial palpi and partial extension of the haustellum. Ordinarily the palpi were folded against the head, and this reaction was quite clear-cut. Touching only the ventral surface of the tarsus of one fore leg with the brush mediated the same response. It was impossible, however, to touch the other tarsi in

FIGURE 2. Head and mouth-parts of an adult caddis-fly (*P. radiatus*) showing the well developed maxillary and labial palpi and the medial haustellum (25 ×).

an intact animal without having the fore tarsi also brought to the brush. The palpi likewise proved to be receptive: touching them with sugar-water elicited spreading of the haustellum. If 1 *M* NaCl solution was used instead of sucrose on the tarsi, there was no reaching with the palpi, and if it was used on the palpi, they were withdrawn sharply.

Further experiments on intact animals were made with glass micro-needles bearing water and sugar-water. The results with the antennae and tarsi were the same as when brushes were used. Touching only the tips of the maxillary palpi with sugar-water elicited reaching with both sets of palpi and partial spreading of the haustellum, much like the reaction obtained by touching the tarsi. Bringing sucrose solution to any part of a maxillary palpus other than the tip of the terminal segment brought about withdrawal, just as with water. Touching the maxillary palpal tips

with NaCl solution elicited a sharp retraction of the palpi. Touching the tips of the labial palpi with sugar-water on a needle elicited spreading of the haustellum in preparation for feeding. Other parts of the labial palpi seemed not to be sensitive, as with the maxillary palpi. With NaCl solution, these palpi were also drawn away. The feeding reaction, therefore, seems to occur in two stages: 1) exploration with the palpi when an acceptable solution touches the tarsi or maxillary palpal tips, and 2) extension and spreading of the haustellum when the solution touches the tips of the labial palpi.

Following these tests, operations were performed to enable us to test parts that could not be touched without interference by known receptors. The forelegs were removed first. Using the brushes, the middle and hind tarsi together were found to be sensitive to sucrose. With care the middle tarsi together or singly could be touched, and this elicited the usual response. With the fore and hind legs removed, the middle tarsi were easily tested and found to bear contact chemoreceptors sensitive to sucrose. With the fore and middle legs removed, the hind tarsi together or singly were also found to be sensitive. All the tarsi, thus, bear the receptors. Using microneedles, the receptors were found on the ventral surfaces of the tarsi and not on the other parts of the legs, but the exact segments of the tarsi bearing them were not determined.

With the last segments of the maxillary and labial palpi removed, the reactions to contact of the palpi with sucrose solutions were abolished, thus confirming previous observations that the receptors were confined to these segments. To test the sensitivity of the haustellum, the fore and middle legs and the palpi were removed, and the animals offered water and sugar-water on brushes. A little difficulty was encountered at first, because the haustellum became covered with clotted hemolymph from the cut ends of the palpi. After this was washed off, however, the animals were able to feed. They obviously could distinguish sucrose solution from water, spreading the haustellum and drinking the former when sated with water. If NaCl was used instead of sucrose, they refused to drink. If they were drinking sugar-water and NaCl solution was suddenly substituted, they reacted by immediate withdrawal of the haustellum and often by violent retraction of the head. Thus the receptors could distinguish acceptable from unacceptable materials in solution. The receptors were not located exactly on the haustellum, but they would seem to be near the distal margins, for application to the tip of the haustellum of a droplet of sugar-water on a needle brought about almost immediate extension.

The parts of the body bearing contact chemoreceptors sensitive at least to sucrose and NaCl, therefore, are the ventral sides of all the tarsi, the tips of the terminal segments of the maxillary and labial palpi and the haustellum. Generally the reaction to appropriate stimulation of the tarsal or maxillary palpal receptors is reaching with and vibration of the palpi and partial extension of the haustellum. The reaction to appropriate stimulation of the labial palpal tips or the haustellum is extension and spreading of the haustellum and feeding. No differences were noted between the reactions of males and females.

The tarsi, palpi and haustellum of these three species were mounted on slides and examined in an attempt to find the possible end-organs involved. On the ventral surfaces of the tarsi there are many short, thin walled, trichoid sensilla among the longer hairs and spines. These are quite similar to the probable receptors in

Lepidoptera and Diptera (Eltringham, 1933; Frings and Frings, 1949; Grabowski and Dethier, 1954; Hayes and Liu, 1947; Lewis, 1954a, 1954b; Tinbergen 1939). On the palpi there are many trichoid sensilla on all the segments, but there is no way at present to select any as possible receptors. On the posterior face of the haustellum, there are basiconic and trichoid sensilla in small numbers. Either of these might be involved, because no other obvious sensilla are present, but the data do not warrant any definite selection. It is probable, therefore, that the receptors on the tarsi and palpi are trichoid sensilla, while those on the haustellum are either trichoid or basiconic.

With *O. cinerascens* only a few tests were made on mounted animals before their untimely deaths. It was obvious that they had tarsal and palpal receptors like the others tested, but these were not further localized. With this species and with *O. americana* many observations were made on unmounted individuals that visited the laboratory table where the others were being tested or came onto the brushes and needles used in the experiments. They scurried about in characteristic, excited manner, turning to and fro, antennae vibrating. If a droplet of water was in their way, they usually stopped as soon as they touched it and drank. When sated with water, they no longer stopped at these droplets. If a droplet of sucrose solution was placed in their way, however, they stopped as soon as the tarsi touched it and turned round and round reaching with the palpi. As soon as the palpi touched the droplet of sugar-water the haustellum was extended and the insect fed.

Discussion

These observations and experiments on adults of six species of Trichoptera representing four families show that, in these at least, the mouth-parts are functional, modified for sucking, and that the adults feed. These results are fully concordant with the reports of Lucas (1893), Siltala (1907) and Döhler (1914). Ulmer (1904) and Cummings (1913, 1914) reported that only a few species from one or two families lack a well developed haustellum. While all the observations on living animals have been made on representatives from only four families and mostly from two families, the conclusions may have validity for Trichoptera generally. At least no one has shown experimentally that any species does not feed in the adult state.

The presence of tarsal contact chemoreceptors also indicates affinity with the haustellate insects. Those studied to date (Hemiptera, Lepidoptera, Diptera, Hymenoptera), in contrast with the mandibulate forms, have contact chemoreceptors on the tarsi (Frings and Frings, 1949). The near certainty that the tarsal and palpal end-organs of Trichoptera are trichoid sensilla further allies them with the typical haustellate forms. Haustellate species, in general, have trichoid sensilla for trophic contact chemoreception and mandibulate forms basiconic sensilla (Frings and Frings, 1949). In the presence of contact chemoreceptors on the palpi and the absence on the antennae, the Trichoptera are more like the Diptera than the Lepidoptera (Frings and Frings, 1949, 1956). The general form of the mouth-parts in Trichoptera also is more like that of Diptera than Lepidoptera. How much weight can be attached to evidence such as this in determining relationships among larger groups of insects we do not know. Certainly, however, further comparative studies of contact chemoreception in Trichoptera, as well as other haustellate groups, would be desirable.

Summary

The loci of contact chemoreceptors sensitive to sucrose and NaCl in solution and mediating feeding responses were determined experimentally in adult Trichoptera of four species from the families, Phryganeidae and Limnephilidae. The receptors are on the ventral surfaces of all the tarsi, the tips of the maxillary and labial palpi and the haustellum. The animals feed on liquids, and these receptors allow them to distinguish acceptable from non-acceptable materials in solution. Less precise observations on two other species from two other families showed a similar situation in these. The end-organs are probably trichoid sensilla. This fact, along with the presence of tarsal contact chemoreceptors, places adult Trichoptera of these species, at least, among typical haustellate insects, most nearly resembling many Diptera in locations of the receptors and feeding reactions.

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THE FIREFLY PSEUDOFLASH IN RELATION TO PHOTOGENIC CONTROL [1]

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Introduction

The normal flashes of many fireflies are short sharp bursts of light lasting about a tenth of a second, with essentially total darkness between. The problem of how this light emission is so precisely controlled has long appealed to investigators interested in biological trigger mechanisms. Two basic facts have become firmly established: that nervous activity can initiate luminescence and that oxygen is essential for light production both in the intact firefly and in cell-free extracts (for review see Buck, 1948; Harvey, 1952; McElroy and Hastings, 1955; Buck, 1955).

There are two principal theories concerning the control of normal flashing. One postulates direct nervous stimulation of the photogenic cell, and the other proposes nervous control of the oxygen supply to the cell. There has been little empirical evidence bearing on the idea that the nerve impulse acts on the photogenic cell directly. The theory of oxygen limitation, on the other hand, has been widely supported on both physiological and anatomical grounds. Actually, as pointed out previously (Buck, 1948), all the experimental findings that have been ascribed to direct oxygen control can be equally well interpreted as effects on nervous control. The anatomical evidence is more persuasive, though circumstantial. It consists of the facts (a) that tracheal end cells under the light microscope appear to have a structure which can be interpreted as valvular (Dahlgren, 1917), (b) that end cells are present in the photogenic organs of flashing types of fireflies and absent in types that produce only sustained glows, and (c) that the end cells are strategically situated on the tracheae at the points where the tracheoles enter the photogenic tissue.

The principal experimental support for the idea that the end cell functions as a valve comes from the work of Snell (1932) and Alexander (1943). They found that fireflies exposed to low oxygen concentrations developed a dull "anoxic glow" (= our "hypoxic glow"), and when suddenly re-exposed to air produced a brilliant "pseudoflash" lasting a second or more. They interpreted these events as follows: The low oxygen narcotizes the normally closed end cell valves and causes them to open, allowing the ambient gas to enter the previously anaerobic photogenic cytoplasm. Since the oxygen concentration is low, only a dull glow develops. When air is subsequently admitted the higher oxygen concentration permits a more brilliant luminescence, which, however, then quickly dies out as the valves recover and close in the higher oxygen concentration. From these hypoxic glow-pseudoflash responses it was argued that normal flashing could also be controlled by end cell limitation of oxygen. It should be noted, however, that all the experiments in-

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volved markedly unnatural conditions and hence are not necessarily relevant to normal flash control.

Recent experiments of McElroy and associates on cell-free extracts of firefly photogenic tissue appear to bear directly on the question of oxygen limitation versus nerve stimulation. When all the components required for light emission (luciferin, luciferase, Mg^{++}, adenosine triphosphate and oxygen) are mixed together a flash of light occurs, its intensity reaching a peak in less than 0.1 second and then declining to a low sustained level within the next 10–20 seconds. Evidence too detailed to present here indicates that this *in vitro* flash involves several reactions (McElroy and Hastings, 1955; McElroy, personal communication). First, it is believed, luciferin, luciferase and ATP react to form a luciferin-adenylic acid-enzyme "active intermediate." The intermediate is then oxidized rapidly and irreversibly, with emission of light. This oxidation corresponds to the initial rise of luminescence in the flash. However, the oxyluciferin formed during luminescence undergoes a slower, reversible phosphorylation by ATP to form oxyluciferin-adenylic acid, which strongly inhibits the enzyme in the active intermediate. This inhibition accounts for the decline in light intensity after the initial peak—a decline which takes place in the presence of excess oxygen. The eventual low level plateau of luminescence thus reflects the low concentration of uninhibited enzyme available once a steady state among the various reactions is established.

The concentration of the active intermediate—which may be considered to be the substrate of the light-producing reaction—can be changed in two significantly different ways: (1) If oxygen concentration is greatly decreased the rate of the oxidative reaction is decreased, resulting in decreased luminescence and accumulation of active intermediate. When air is readmitted the accumulated intermediate is rapidly oxidized, resulting in a flash of light. This shows that it is possible, by changing oxygen concentration, both to limit luminescence and to cause a flash. The "oxygen flash" of the extract has a remarkable quantitative resemblance to the pseudoflash of the intact firefly, which, it will be remembered, is induced by a similar sequence of changes in oxygen concentration. This suggests that both types of flash are due to oxidation of accumulated active intermediate, and that neither of them needs be oxygen-limited during its decay phase. (2) The concentration of the active intermediate may also be increased by addition of pyrophosphate, which, by opposing the formation of the oxyluciferin-adenylic acid inhibitor, frees active enzyme. Since this reversal of enzyme inhibition is rapid, lasts only until the added pyrophosphate is used up, and does not involve any change in oxygen concentration, it provides a possible model for the mechanism which induces the normal flash.

The *in vitro* system therefore suggests that the flashing of the firefly need not normally be controlled by oxygen concentration even though light production may, under some artificial conditions, become oxygen-limited. In view of this possibility, and of the ambiguity of previous oxygen-limitation experiments on intact fireflies, it is important to re-examine the evidence purporting to demonstrate end-cell control of luminescence.

Materials and Methods

The forms investigated were adults of the lampyrid fireflies *Photuris* sp. and *Photinus pyralis* from the Baltimore-Washington area, adults of the elaterid firefly

Pyrophorus atlanticus from Florida, and larvae of *Photuris*. In the males of the first two species, as in many lampyrid fireflies, the photogenic tissue occupies the ventral surfaces of abdominal segments 6 and 7. In *Pyrophorus* we investigated the small circular organs at the posterior dorsal corners of the prothorax. In the *Photuris* larva the photogenic organs are a pair of small lateral plaques on the ventral side of abdominal segment 8. Similar organs exist in the larva and pupa of *Photinus pyralis*, and sometimes persist into the adult where they function independently of the main organs.

Different gas mixtures were prepared by passing various gases through calibrated flow meters into a mixing chamber and thence to the exposure chamber, which was a 5 cm. length of glass tubing of 6 mm. bore. For visual observation up to three specimens were accommodated in the chamber, separated by wire screen partitions. A flow rate of 300–400 mL per minute was used and a reversing stopcock between mixing and exposure chambers permitted quick shifting from one gas to another, the unused gas being vented. Light intensity was measured with a photomultiplier tube apparatus (Hastings, McElroy and Coulombre, 1953) and recorded with an oscilloscope camera or a graphic meter, the firefly being held in position against the wall of the exposure chamber with a loose cotton plug.

Results

1. Responses of intact lampyrid fireflies to varied oxygen concentration

When adults of *Photinus* and *Photuris* were exposed to various low oxygen concentrations a dim hypoxic glow usually developed, and when such glowing fireflies were suddenly re-exposed to air a pseudoflash occurred. Figure 1 illustrates a response of this sort, the hypoxic glow being represented by BC and the pseudoflash by CDE. Figure 2 shows the pseudoflash portion in more detail. An "oxygen flash," which occurs when air is readmitted to an anaerobic cell-free extract of photogenic tissue, is reproduced in Figure 3 for comparison. About 50 records of the hypoxic glow and pseudoflash have been made and analyzed, supplemented by many hundred visual observations. These in general confirm the findings of Snell and of Alexander, but certain differences were noted. For example, if a firefly was left in the low oxygen mixture after the hypoxic glow had reached its plateau level, instead of then exposing it to air, the intensity of the glow usually decreased over the course of several minutes (Fig. 1, dotted line, FG). It was also observed that some individuals failed to give a pseudoflash, or both hypoxic glow and pseudoflash, and that there was considerable variation in the length of the period between hypoxic exposure and beginning of the hypoxic glow (Fig. 1, AB), and in the intensities of both glow and pseudoflash. Also, as in nature, normal individuals sometimes showed an initial constant dim glow in air which made it difficult to recognize the start of the hypoxic glow.

In spite of these variations in response, a number of quantitative relations were apparent. First, in fireflies which had been exposed to a particular low oxygen gas mixture, the peak intensity of the pseudoflash in air was approximately proportional to the intensity of the hypoxic glow in the low oxygen mixture just prior to admission of air. Before the beginning of the hypoxic glow no pseudoflash could be elicited; during the dimmer periods of the hypoxic glow, either before or after the maximum, pseudoflashes of low intensity occurred; and during the brighter pe-

riods of the hypoxic glow more brilliant pseudoflashes occurred. In 35 experiments in which the hypoxic glow was induced with 0.25% oxygen, the ratio of pseudoflash intensity to hypoxic glow intensity varied only from 30 to 120, and the range for repeated measurements on single individuals was even smaller. Thus both pseudoflash and hypoxic glow intensities pass through a maximum with time.

A second finding was that the ratio of pseudoflash intensity to hypoxic glow intensity varied with the oxygen concentration used to induce the hypoxic glow. For example, in one typical individual the pseudoflash intensity in air was 1.5 times

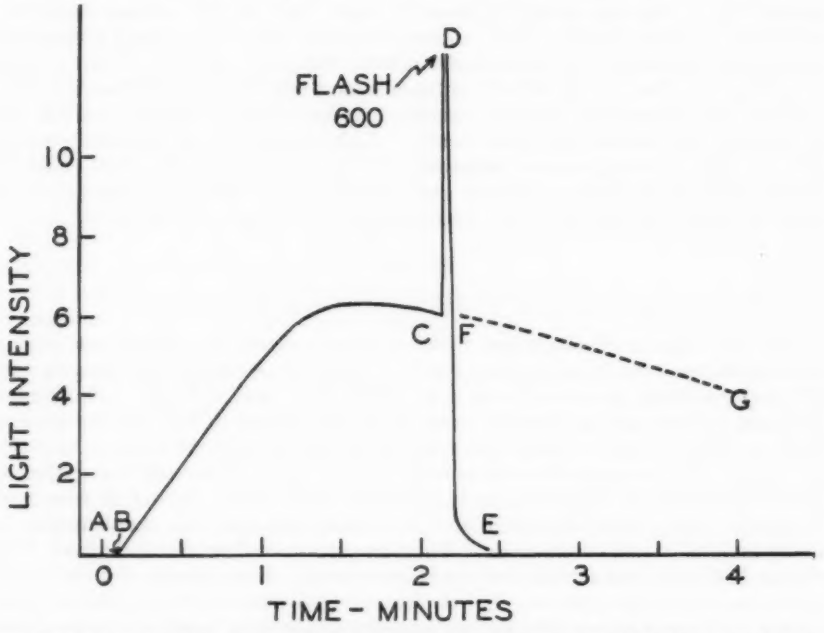
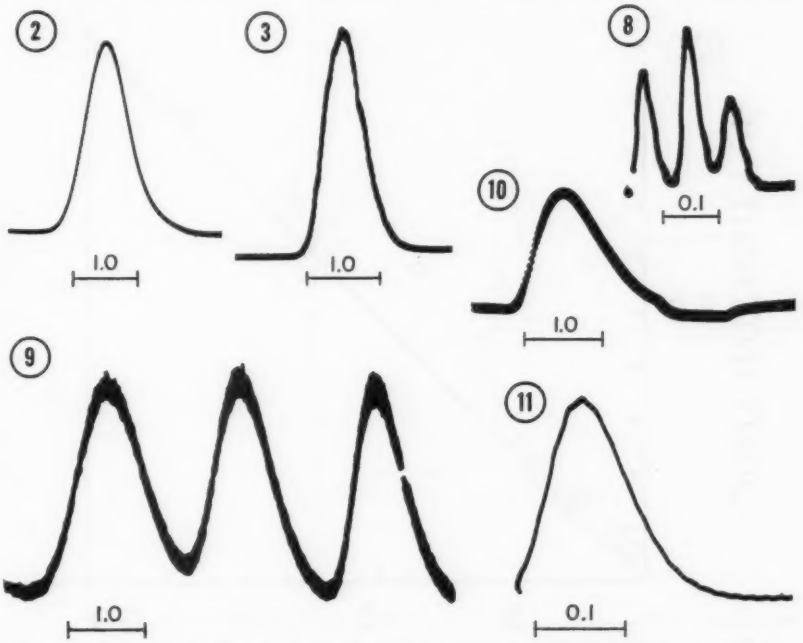


FIGURE 1. Hypoxic glow-pseudoflash response of intact male of *Photinus pyralis*, diagrammed from graphic meter record. Ordinate, light intensity in arbitrary units. At A (zero time) ¼% oxygen was introduced. At B the hypoxic glow began, AB representing the latent period. At C air was flushed through the chamber and a pseudoflash of 600 units relative intensity occurred. Had the firefly been left in ¼% oxygen at C, the hypoxic glow would have continued (FG), slowly diminishing in intensity.

as great as the hypoxic glow elicited with 2.4% oxygen;[2] when 1% oxygen was used the pseudoflash was 10 times as bright as the glow; when 0.25% oxygen was used the pseudoflash was 60 times as bright; and when 0.05% oxygen was used the pseudoflash was 5000 times as bright. The increase in this ratio with progressively lower oxygen concentrations was evidently due both to diminution in hypoxic glow intensity and to an increase in the absolute pseudoflash intensity.

[2] No systematic attempt was made to find the upper oxygen concentration limit for pseudoflash occurrence, but it is certainly higher than the limit set by Snell (about ½%).

A third characteristic of the hypoxic glow-pseudoflash response is that the pseudoflash was remarkably constant in duration and in form (rates of accretion and decay of intensity), regardless of variations in both degree and duration of hypoxia prior to readmission of air. This was true both in repeated measurements with one individual and in records from different individuals. Intensity variations of well over a thousand-fold occurred without difference in duration. If the pseudoflash were being controlled by some sort of oxygen-sensitive effector, such as Snell



FIGURES 2, 3, 8, 9, 10, 11. Various luminous responses photographed from the oscilloscope screen. Ordinate, light intensity; abscissa, time, with sweep going from left to right. Further descriptions in text. Time scale in seconds. FIG. 2. Pseudoflash of intact adult male of *Photinus pyralis*. FIG. 3. Oxygen flash of cell-free extract of *P. pyralis*. FIG. 8. Spontaneous flashing of intact female of *Photuris*. FIG. 9. Spontaneous glow of intact *Photuris* larva. FIG. 10. Pseudoflash of *Photuris* larva. Temporary depression in trace following flash represents period during which photocell power supply was switched off. FIG. 11. Spontaneous flash of intact male of *P. pyralis*.

supposed the end cell to be, it would be remarkable that this degree of constancy of response could be achieved, particularly in view of the individual variability in intensity and latency of hypoxic glow, and in intensity of pseudoflash.

2. Oxygen responses in relation to structure

It was shown previously (Buck, 1948) that there is no correlation between the state of spiracular valves and the times of occurrence or characteristics of the normal

flash of *Photinus pyralis*, and that when intact fireflies are tested with progressively falling oxygen concentration the spiracles open well before the hypoxic glow begins and close after it ceases. Evidence that the spiracles have no immediate influence on the hypoxic glow-pseudoflash response was obtained in the present study by testing adults of *Photuris* and *Photinus* in which the spiracles of the luminous segments had been made inoperative by cautery with an electrically heated needle or by insertion of a short length of human baby hair. Although these specimens often showed a continuous glow in air, presumably caused by the mechanical dis-

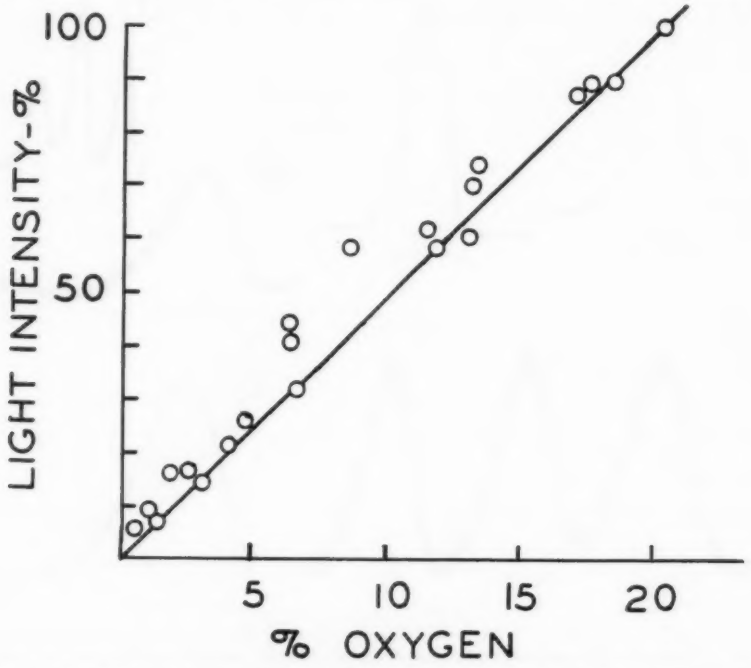


FIGURE 4. Relation between oxygen concentration and glow intensity (in per cent of intensity in air) of smeared photogenic tissue of males of *P. pyralis*. Data from several experiments.

turbance, they gave pseudoflashes similar to those in individuals with normal spiracles. Likewise, it was observed that spiracular opening is regularly induced by exposure to 5% oxygen, whereas the hypoxic glow usually requires that the ambient oxygen concentration be reduced to the order of 1% to 2%. Absence of spiracular or indeed any sort of valvular influence is also seen in dead fireflies which, if prevented from drying out, may exhibit a constant dim air glow for a day or more after all visible signs of life have disappeared. Such dead specimens have permanently open spiracles yet give a pseudoflash response.

A series of experiments was performed in which increasing degrees of interfer-

ence with possible central nervous or tracheal control of luminescence were achieved by (a) decapitation, (b) cutting off the abdomen at the junction of the fifth and sixth segments, (c) excising the photogenic organ alone, and (d) smearing the photogenic tissue on glass. None of these preparations produced normal flashes, or indeed any continued spontaneous luminescence, except for the smeared organs, which exhibited a continuous dim glow in air, decreasing in intensity very gradually

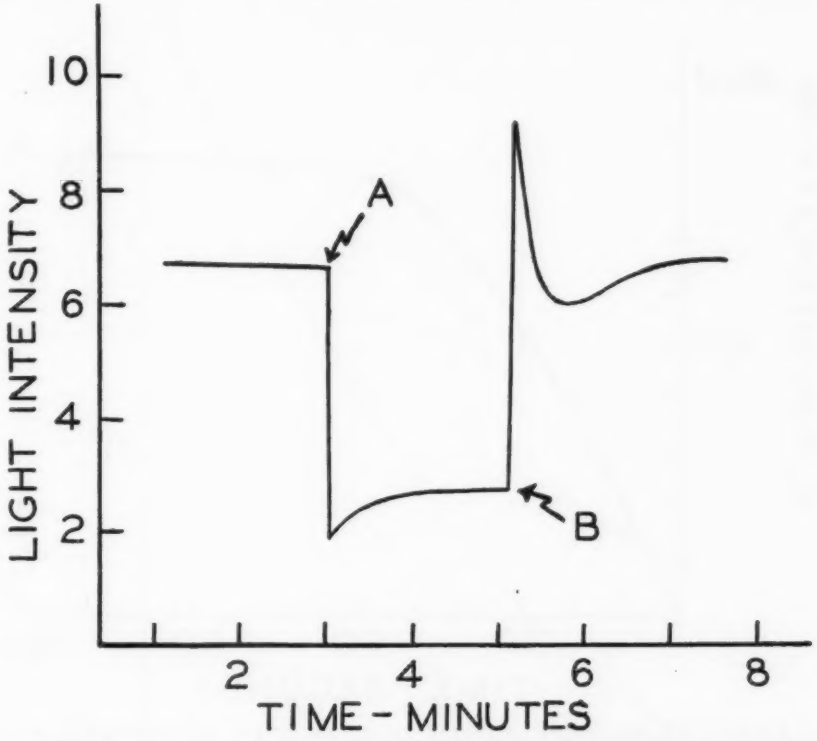


FIGURE 5. Changes in light intensity of smeared photogenic tissue of male of *P. pyralis* in varied oxygen concentrations. Tissue in air for first three minutes. At A, 8.3% oxygen was introduced. At B, air was readmitted, inducing a pseudoflash-like excess luminescence.

(30 minutes or more to extinction). The first three types of preparations responded to changes in ambient oxygen concentration just like intact fireflies, *i.e.*, in low oxygen concentrations they developed hypoxic glows and when air was readmitted they produced typical pseudoflashes. It is thus clear that nerve impulses originating in the central nervous system play no role in the photogenic response to hypoxia. It is also apparent that none of the tracheae external to the light organ is involved.

The intensity of the glow of the smeared organ is proportional to ambient oxy-

gen concentration below 21% (Fig. 4) and also increases greatly in pure oxygen. Figure 5 shows the time course of the luminescence when a smeared organ was exposed (at A) to 8.3% oxygen and then re-exposed (at B) to air. The changes in luminescence are qualitatively very similar to those which occur when the same procedure is carried out with cell-free extracts (Hastings, McElroy and Coulombre, 1953). The smeared preparation differs from both the extracts and the intact organ in that its pseudoflash has a longer duration and is not so bright relative to the

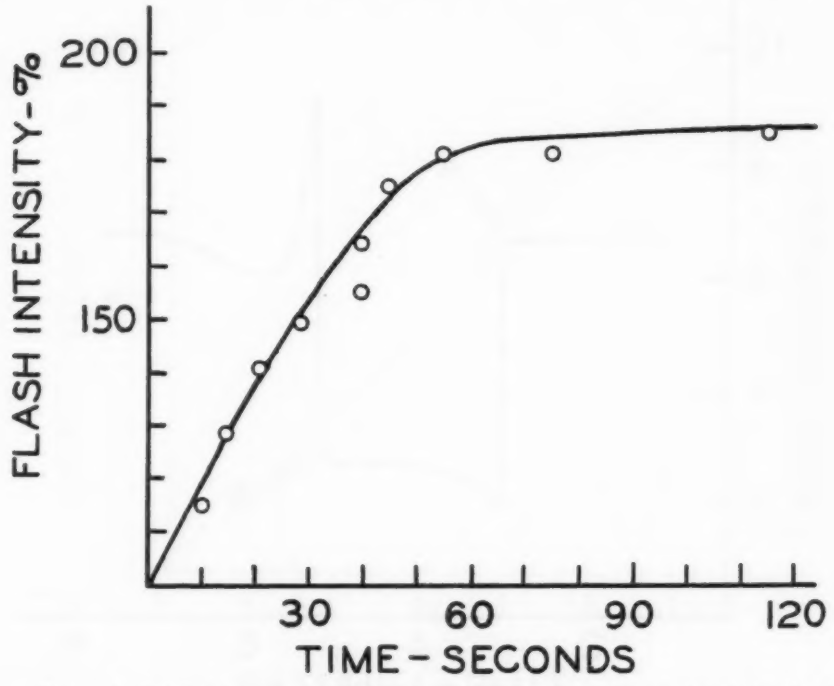


FIGURE 6. Relation between duration of hypoxia and intensity of pseudoflash in air of smeared photogenic tissue of *P. pyralis*. Each point represents pseudoflash intensity, in per cent of glow intensity in air, in an experiment similar to that diagrammed in Figure 5. The hypoxic mixture used contained 1% oxygen.

intensity of the hypoxic glow. These differences in glow and flash are to be expected if oxygen has become limiting in the luminescent reaction, and presumably it is the disruption of the tracheal supply within the photogenic tissue itself which is responsible for this oxygen-limitation. Similar "slow" flash responses have been demonstrated in extracts under conditions of oxygen limitation (McElroy and Hastings, unpublished). On the basis of the biochemical reactions already discussed, the intensity of the pseudoflash of the smeared organ is presumably a measure of the amount of active intermediate which has accumulated. The de-

pendence of this accumulation upon both time of hypoxia and oxygen concentration in the hypoxic gas mixture is illustrated in Figures 6 and 7.

In normal males of *Photinus* and *Photuris* all the photogenic tissue in both luminous segments ordinarily participates in each flash, and apparently simultaneously.

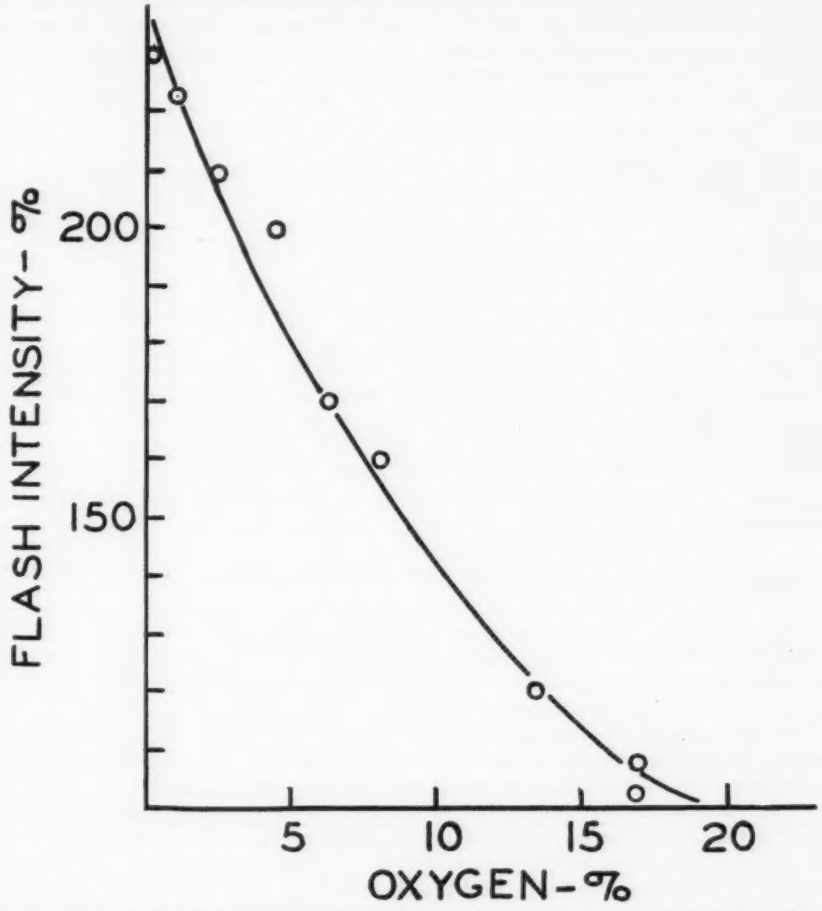


FIGURE 7. Relation between intensity of pseudoflash in air of smeared photogenic tissue of *P. pyralis*, and degree of hypoxia. Tissue exposed to each oxygen concentration for two minutes, then flushed with air. Intensity plotted on ordinate in per cent of glow intensity in air.

In some instances, however, it was observed that both spontaneous flashes and various types of induced luminescence involved only one of the segments, or only parts of one or both. Furthermore, the type of luminescence displayed sometimes differed in different regions of a single organ or even changed in the course of an experiment.

The flash of the female of *Photuris* is usually too sharp and brilliant for reliable visual observation of heterogeneity, but may possibly also involve asynchronous luminescence (Fig. 8).

In instances in which only a portion of a photogenic organ gave the hypoxic glow-pseudoflash response, the portion which failed to respond often developed a dull glow in air after the pseudoflash in the other portion had ceased. Since the photocell integrates all the light emitted, one needs to be aware of the possibility that intensity × time recordings of luminescence (*e.g.*, Figs. 1, 2) may be the resultant of two quite different sorts of things, namely, change in light intensity per unit organ area, and change in area active. We cannot exclude the possibility that an occasional heterogeneous response of this type was recorded in our work, but do not believe that any of our present interpretations is in error because of such an accident.

3. Oxygen effects on induced glows

Bright steady glows can be induced in both intact fireflies and isolated abdomens by air passed through cotton soaked in ethyl ether or over potassium cyanide crystals (*i.e.*, without change in ambient oxygen concentration). The dosage must be chosen to avoid either premature recovery of the animal or rapid destruction of the photochemical system (Buck, 1948). When fireflies which were glowing from exposure to ether or cyanide vapor were exposed to low oxygen concentrations, luminescence abruptly declined to a low level, then rose somewhat as an hypoxic glow. When air was readmitted a typical pseudoflash occurred. This illustrates the occurrence of a flash under conditions in which tracheal end cells would be expected to be inactivated.

4. Oxygen responses of Pyrophorus, Photuris larva and persistent pupal organ of Photinus pyralis

The photogenic organs of the large elaterid firefly *Pyrophorus* and of the larvae and pupae of lampyrid fireflies offer an interesting contrast to the organs of adult lampyrid fireflies in two respects. First, they never normally flash, but emit light in long-sustained glows at irregular intervals. Second, they lack the tracheal end cells which are characteristic of the flashing-type adult lampyrid organ. The pupal organs frequently persist into the adult, thus combining both organ types in the same individual.

Observation of oxygen effects in *Pyrophorus* is complicated by the fact that the glow normally fluctuates cyclically in intensity, at frequencies varying from about one peak per second to one per five seconds or slower, as observed also in a Cuban species by Harvey (1931). In addition, the intensity of the glow increases markedly when the creature is disturbed. Thus hypoxia sometimes proves sufficiently irritating that the light emitted is at first actually brighter than in air, and there is no initial decline due to oxygen limitation as in adult lampyrids glowing in air. The luminescence is only oxygen-limited at ambient concentrations of 1% or lower. *Pyrophorus* in low oxygen concentrations responds to a sudden increase in oxygen by emitting a pseudoflash which is qualitatively similar to the typical adult lampyrid pseudoflash, but often appears after a quite long latent period (up to 20 seconds) and lasts much longer.

The minute photogenic organs of the larva of *Photuris* and the persistent pupal organs of *Photinus pyralis* emit a fluctuating luminescence strikingly similar to that of *Pyrophorus* (Fig. 9). Their responses to oxygen have not been followed in detail except to confirm Buck's observation (1946, 1948) that low ambient oxygen induces an hypoxic glow and subsequently raised oxygen elicits a pseudoflash. A record reproduced in Figure 10 shows that the larval pseudoflash closely resembles that of the adult. The persistent pupal organ in the adult usually (but not always) gives an hypoxic glow-pseudoflash response in parallel with that of the adult organ.

Discussion

Evidence presented above has shown that neither spiracle, main trachea nor central nervous system is necessary for either the appearance or disappearance of luminescence in the usual type of hypoxic glow-pseudoflash response. The possibility that tracheal end cell valves might be involved in the response is likewise all but eliminated by the following considerations: (a) Pseudoflashes occur in *Pyrophorus*, the *Photuris* larva and the persistent pupal organ of *Photinus pyralis* (all of which lack end cells), and in lampyrid fireflies treated with cyanide and ether (where end cell valves should be inactivated); (b) as already pointed out, the constancy of pseudoflash duration makes it difficult to believe that an end cell mechanism is functioning in the control; (c) recent electron microscopy by Beams and Anderson (1955) casts grave doubt on there being any valvular structure in the end cell. In fact the induction of pseudoflashes in dead fireflies makes it unlikely that this response depends upon active participation of any part of either tracheal or nervous systems.

Even with end cell control excluded there remains the question of whether the hypoxic glow-pseudoflash response might nevertheless be controlled by oxygen limitation. We have seen that in the glow of smeared tissue and, within a narrow concentration range, in the hypoxic glow itself, oxygen does appear to be a limiting reactant. However, the hypoxic glow is actually induced not by increase in oxygen concentration but by decrease, and the pseudoflash dies away (*i.e.*, is controlled) under conditions in which ambient oxygen concentration, if changing at all, must be rising. When we add to these paradoxes the fact that glowing can be induced by pure oxygen (Alexander) and by a wide variety of physical and chemical agencies, and that even the hypoxic glow can change spontaneously in intensity without any change in ambient gas concentration, it becomes very difficult to visualize oxygen as playing any consistent role in either initiating or stopping these induced types of luminescence.

For reasons discussed by Buck (1955) the striking kinetic similarity between pseudoflash and oxygen flash (Figs. 2, 3) does not necessarily indicate the same causation. However, the detailed parallels between the two responses leave little doubt that the pseudoflash of the intact organ involves the photochemical system identified in the cell-free extract. Thus active intermediate can be presumed to accumulate in the photogenic tissue during hypoxia, and, upon readmission of air, to be concurrently oxidized and inhibited with production of a pseudoflash (see Introduction). Similarly, assuming that liberation of active intermediate would continue in the organ of a dead firefly until autolysis supervened, the ability of some dead individuals to glow and to give pseudoflashes could be explained. Furthermore, the *in vitro* system is free from all the morphological objections to end cell

involvement discussed above and it is consistent with the constant duration of the pseudoflash, which is particularly difficult to account for on the basis of valvular control. Such constancy, in other words, is precisely what would be expected if the luminescence decays primarily because of an enzyme-inhibiting reaction rather than because of oxygen limitation.

The conclusion that no end cell valve functions in the hypoxic glow-pseudoflash response does not, of course, exclude the possibility that normal flashing is controlled by such a mechanism. Since, however, the induction of the pseudoflash has been the principal experimental support for belief in end cell control, the existence of a more reasonable alternate explanation of the pseudoflash leaves little ground for favoring end cell involvement in the normal flash. Furthermore, oxygen control by whatever method appears intrinsically less tenable than enzymatic control on at least two counts: First, if the flash were oxygen-limited the photogenic tissue would have to be hypoxic throughout the long interflash periods. This would be unlikely on physiological grounds, even if respiration had a lower oxygen requirement than luminescence (actually, in all forms thus far studied luminescence persists at oxygen concentrations far lower than will support any significant respiration). If, however, the normal flash were controlled by temporary reversal of enzyme inhibition, rather than by oxygen limitation, the tissues could remain fully aerated at all times. Second, the normal flash of many lampyrid fireflies is so short as to cast doubt on the possibility of control by diffusive gas transfer. Even in the relatively slow flash of *Photinus pyralis* (Fig. 11) the average rise time is 0.075 second, and the response of individual photogenic units is almost certainly much faster (Buck, 1948, p. 446). In the female of *Photuris* (Fig. 8) the rise time is not more than 0.03 second and the decay of luminescence is almost equally rapid. This shows that an efficient mechanism for oxygen removal would need to be present as well as one for suddenly supplying oxygen. *A priori*, therefore, it would be expected that an intracellular mechanism involving enzyme inhibition and activation would be better suited to the required response velocities than one involving passage of oxygen between tracheae and cell.

The fact that intact fireflies are able to extinguish their light completely between flashes, whereas low-level luminescence continues in the *in vitro* system, need not be unduly disturbing since the intact cell presumably has more efficient methods of shifting the chemical equilibria concerned and of sequestering reactants. It has been suggested (Buck, 1948, 1955) that the photogenic tissue is ordinarily kept dark by some sort of aerobic metabolic process and that light is produced only when this process is interfered with. Such a mechanism would account for both "abnormal" and normal luminescence, since it should be inhibited by very diverse agencies such as hypoxia, various poisons (*e.g.*, pure oxygen, cyanide) and anesthetics (ether)—allowing light to be produced—while at the same time forming a likely type of system to be integrated with the normal biological trigger, the nerve impulse. A tentative biochemical pathway has recently been suggested (McElroy and Hastings, 1955) by which a nerve impulse might lead to a rapid temporary increase in active intermediate concentration—*i.e.*, to a flash. Whether or not the precise mechanism suggested proves to be correct, this general type of linkage of stimulatory and response systems deserves special attention because it provides an endogenous mechanism capable of the observed rapidity and precision of photogenic control.

Summary

As reported by Snell and Alexander, lampyrid fireflies exposed to oxygen concentrations of the order of 2% or lower develop a sustained "hypoxic glow," and when subsequently re-exposed to air emit a much brighter and shorter "pseudoflash." We find that these responses can be independent of the spiracles, and are given by decapitated fireflies, isolated abdomens and excised photogenic organs, showing their independence of central nervous system and tracheae. The hypoxic glow-pseudoflash response is also given by the elaterid firefly *Pyrophorus* and by the larval and pupal photogenic organs of lampyrid fireflies. Since all these organs lack tracheal end cells, these cells cannot, as Snell and Alexander believed, control this type of light production. This, together with other evidence, makes it clear that luminescence is rarely oxygen-limited. Rather, all our observations are consistent with enzyme activation and inhibition in a system of photochemical reactions of the sort proposed by McElroy and Hastings (1955).

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HOW SEA STARS OPEN BIVALVES[1]

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The damage inflicted upon the oyster and clam industries by sea star predation (Galtsoff and Loosanoff, 1939; Barnes, 1946) has stimulated much interest in the method employed by asteroids to open the shells of bivalve molluscs. The many solutions proffered in the past were reduced to two probable alternatives within the last sixty years: (1) the "toxin" theory which proposes that sea stars secrete a substance which produces relaxation of the adductor muscles of their victims; and (2) the "mechanical" theory which credits the sea stars with the ability to pull the valves of the molluscan shell apart by means of their tube feet.

The first hypothesis was proposed originally by Eudes-Deslongchamps (1826). Most of its advocates (including Hesz, 1878; Figuier, 1891; Pieron, 1913; Cahn, 1950; Korringa, 1953 and Aldrich, 1954) postulated that the chemical agent was secreted by the digestive organs of the sea stars. Van der Heyde (1922) and Sawano and Mitsugi (1932) supported this view with experiments which demonstrated that extracts of asteroid stomach and/or pyloric caeca produce tetanus and, often, permanent cessation of cardiac beat when poured over the hearts of living molluscs.

The mechanical theory, advanced originally by Fischer (1864) and Bell (1892), was established firmly by Schiemenz (1895) who demonstrated experimentally that the valves of the clam *Venus verrucosa* could be separated by a pull of 900 grams, while a clam held by the tube feet of an *Asterias* could be released only if a pull of more than 1000 grams was applied to it. He concluded that the sea star could exert a pull greater than that which could be sustained by *Venus*, but he failed to note that he had measured only the adhesive capacity of the echinoderm's tube feet. He did not show that the sea star possessed the ability to produce sufficient muscular force to open bivalves. However, it is believed that the data presented below demonstrate the existence of such forces and render the toxin theory less tenable.

Materials and Methods

The two groups of experimental procedures employed were designed to determine (1) the effects of sea star extracts upon a representative bivalve, and (2) whether sea stars actually pull upon the valves of their prey.

1. *Procedures for determining effects of extracts*

The stomach and/or pyloric caeca of *Asterias forbesi* (obtained from the Marine Biological Laboratory at Woods Hole) were excised and ground with a Pyrex glass

[1] This investigation is a portion of a dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Zoology at Syracuse University in September, 1955.

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homogenizer in the cold. Enough sea water or distilled water was added to make up 10% solutions relative to the wet weight of the organs used. (Other concentrations were tested and, generally, yielded similar results.) Extraction was allowed to proceed for varying times (5 minutes to 48 hours) and the tissue debris was removed by filtration or centrifugation. Other extraction methods were employed to test the possibilities that the alleged toxin might be only poorly soluble in water, that it might occur in bound form, or that it might require activation. Thus, some extractions were made with fat solvents, some extracts were dialyzed, others were frozen and thawed before use, and some were mixtures of homogenates from different organs.

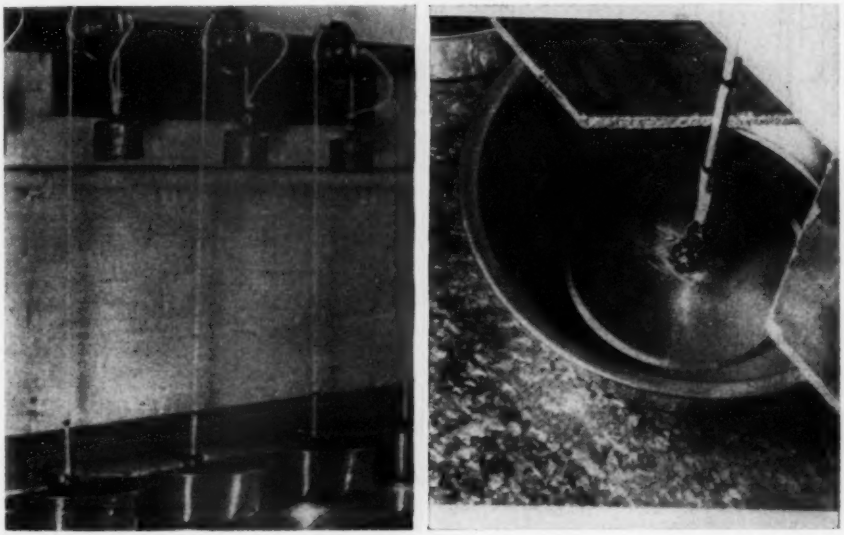

FIGURE 1. Constant stress apparatus. Each 800-gram weight was suspended by a cord passing over a ball-bearinged pulley to a double hook inserted into notches filed in the beak of the mussel shell. Another hook, also made from two bent pins, was soldered to the bottom of the pan and passed through the same notches. Gapes were measured by means of a calibrated metal triangle which could be slipped in between the valves near the hooks.

All extracts were tested on the common sea mussel, *Mytilus edulis*. In most cases 0.5 ml. of the clear extract was injected by means of hypodermic syringe into the mantle cavity or 0.15 ml. was injected directly into the posterior adductor muscle by way of a notch filed in the shell's dorsal edge. Each mussel had been pre-tested to insure that its physiological condition was approximately comparable to that of the other experimental animals. The pre-test was accomplished by exerting a pull of 800 grams on the valves for five minutes; only mussels which gaped less than one mm. were used for injection tests. After being injected, each mussel was subjected to a steady pull of 800 grams on its valves (Fig. 1) for 45 minutes during which measurements of the gape were made at regular intervals.

In some cases, the extract was merely added to the sea water into which the mussel was placed after having been kept out of water for 12 hours, and the gape was determined after 5 and 10 minutes. In other experiments, the mussel heart was exposed and perfused with the extracts while kymograph records were made of the effects on the beat. Controls for all types of tests were treated with solvent only (sea water or distilled water) or with extracts of other sea star organs or extracts of the digestive organs of other invertebrates.

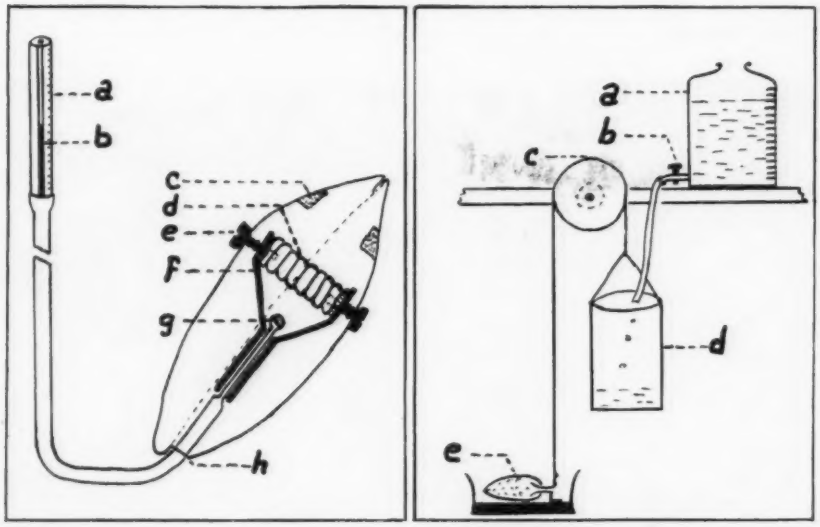

FIGURE 2. Apparatus for measuring sea star pulling force. The device and the mussel are represented at approximately actual size. *a*. calibrated capillary tube; *b*. water column; *c*. cut posterior adductor muscle; *d*. steel coil spring; *e*. bolt; *f*. metal plate soldered to the spring; *g*. plugged end of water-filled rubber tube; *h*. cut umbo. In some experiments this manometric unit was replaced by a plastic cylinder which fitted between the two bolts.

FIGURE 3. Increasing load stress apparatus. *a*. calibrated water jar; *b*. control valve; *c*. pulley; *d*. waxed cardboard container; *e*. mussel. The approximate total load applied to the shell was computed by adding the container weight to the weight of the water poured into it from the calibrated jar.

2. *Procedures for determining sea star pulling ability*

The adductor muscle of medium-sized *Mytilus* was severed with a thin razor blade and the valves were then made to shut firmly by means of an "artificial muscle." This consisted of a tightly coiled steel spring about ½ inch long with a metal plate soldered at each end. The spring was held in place (Fig. 2) by short bolts inserted through holes bored in the valves. The metal plates were bent so as to compress the sealed end of a water-filled rubber tube which passed out of the shell through a hole effected by breaking off one tip of the umbo. The distal end of the rubber tube was slipped over the end of a graduated capillary tube. Any outward

pull on the valves was reflected in the stretching of the spring and, consequently, in an increased volume of the rubber tubing and a lowering of the water level in the manometer tube. The variations in water level, produced by a sea star humped over a mussel containing this apparatus, could be duplicated by inserting the mussel, afterward, in the stress apparatus illustrated in Figure 3. In some instances, the severed adductor muscle was replaced by a threaded plastic cylinder so that the valves could be bolted together firmly or allowed to separate only slightly.

TABLE I

Gaping of Mytilus under stress. These raw data are from two representative groups of experiments involving the application of stress to the shells after injections into the adductor muscles. The apparatus permitted the testing of 10 mussels simultaneously; generally, five were treated with extract and five with control solutions. Shells ranged in size from 43 × 22 mm. to 55 × 30 mm.

Injected with	Gape in millimeters after					
	5 min.	10 min.	15 min.	20 min.	25 min.	45 min.
Distilled water	0	0	0	0	0	0
	0	0	0.7	2.0	0.2	2.0
	0	0	4.8	4.0	5.8	7.8
	0.5	0	9.6	0.2	2.8	2.2
	0.9	2.8	1.7	1.8	2.8	1.8
	2.2	2.5	2.7	2.7	2.5	2.5
	2.8	2.9	3.8	3.0	3.8	3.2
	3.5	3.2	3.2	3.8	3.5	4.0
	3.8	4.8	4.9	5.0	5.0	5.5
	3.8	4.0	3.8	4.8	5.7	6.2
Pyloric caeca in distilled water	0	0	0	0	0	2.2
	0	0	0	0	0	4.8
	0	0	0	0.8	3.7	4.8
	1.8	1.8	1.9	3.0	3.0	2.8
	1.7	3.0	3.2	3.0	3.0	2.0
	1.8	1.8	1.8	1.8	1.9	5.8
	2.1	2.1	2.1	2.2	2.5	3.0
	3.8	3.8	3.8	5.0	5.3	5.4
	4.0	4.0	3.8	4.0	4.8	4.8
	4.8	5.7	5.8	5.8	5.8	4.8

RESULTS

1. *Effects of sea star extracts*

As reported by previous investigators, extracts of the digestive organs of sea stars generally produce tetanus in molluscan hearts. But so do other substances including sea water. Furthermore, any suggestion that an asteroid secretion may affect the adductor muscle indirectly by stopping the heart seems untenable in view of the observation, made in some of these experiments, that *Mytilus* whose hearts are excised may continue to maintain their valves tightly shut for two or three days.

Mytilus placed in sea water contaminated with sea star extracts usually "taste" the medium and then close their valves firmly. The degree of gaping, among the

sixty mussels tested in this manner, was less for specimens exposed to diluted extracts than for those placed in sea water alone. This seems to indicate that no muscle-relaxing toxin was present in the extracts.

Gape measurements made on mussels injected with extracts or control solutions revealed that the rate of shell opening varied through a very narrow range for all tests. The average value of the rate of gaping for mussels which were not injected was almost identical to that of mussels whose mantle cavity or adductor had been injected with sea water or distilled water or with one of the various types of extracts (see representative data in Table I). Over 1000 mussels were tested in

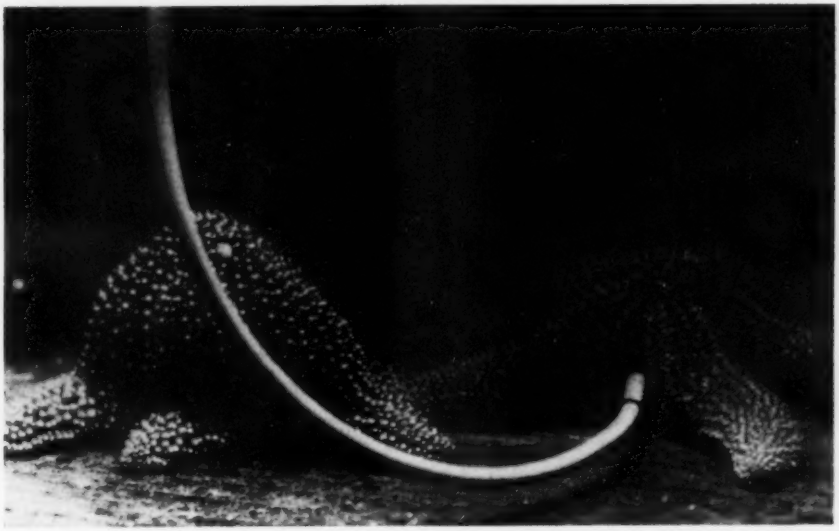

FIGURE 4. *Asterias* feeding upon decoy mussels. The rubber tube leading to the manometric recorder is covered with a glass sleeve near the mussel in order to prevent compression of the tube by the sea star's antimeres. The asteroid on the left is in the process of inserting its stomach into a shell whose valves are tightly bolted together by means of plastic cylinder.

this manner and the data can only lead to the conclusion that the extracts did not contain any substance which could be considered effective in inducing relaxation of the bivalve adductor muscles.

2. Observation of sea star pulling ability

Sea stars, kept in 20-gallon tanks of circulating sea water, were presented live mussels whose adductors had been replaced by springs or cylinders as described previously. The soft parts of most of these mussels were reached by the asteroid stomachs and were partly or wholly digested. Unquestionably, no secretion of the sea stars could have had any weakening effect upon the "artificial muscles" holding the pelecypod valves closed. The following cases, selected from several dozen observations, illustrate the significance of the results obtained:

1) A sea star was observed while it approached a mussel containing the spring device and while it humped over its victim in the typical predatory position (Fig. 4). During the five minutes it required to settle in an advantageous position (and, probably, to extrude its stomach) there was no change in the water level of the recording apparatus. During the next three minutes, however, the level dropped rapidly; at the end of this time the sea star was removed from the aquarium and its arms were peeled back forcibly in order to expose the mussel. The valves were found closed tightly upon the sea star's stomach, most of which was inserted into the shell. In this case, the drop in the recording tube was duplicated later with a load of 1200 grams on the shell's valves; but spring-containing mussels requiring 2600 to 3000 grams pull to open 0.1 mm. were also successfully preyed upon by the sea stars.

TABLE II

Summary of pulling forces exerted by a sea star upon a spring-containing mussel

Time (in minutes) from beginning of observation	Pulling force (in grams) applied by the sea star
0	440
5	740
10	620
14	710
15	560
20	620
60	470
90	0
115	650
135	0
150	680
155	800
158	0
159–165	Sea star moved off mussel

2) Another *Asterias* was observed for almost three hours after it was found humped over a prepared mussel. During that time, the water level of the recording tube varied through three irregular cycles of rises and falls. When these variations were duplicated later by placing the mussel in the stress apparatus, it was seen that they represented the pulling forces shown in Table II. When the mussel was opened it was found to be partly digested. This, and many similar observations, seems to indicate that the sea star's pull is not applied steadily.

3) A mussel whose valves were bolted together very firmly so that no space could be discerned between them under 9 × magnification, was loosened forcibly from the grasp of a sea star that had humped over it for several hours. The asteroid's stomach was mostly inside the shell and it did not slip out again during the next hour while the sea star dragged the shell along the bottom of the aquarium. Later, when the shell was exposed to increasing loads in the stress apparatus, the valves were bent enough by a load of 3100 grams to produce an opening between them of 0.1 mm.

4) Several mussels whose valves were tied together so as to open only 0.1 mm. were invaded by sea stars whose stomachs were seen to slip out of the shells when the echinoderms' arms were pulled away from the shells.

Discussion

The negative results of the experiments involving sea star extracts are not proof that asteroids do not secrete a toxin during predation, but they do indicate that no such substance can be separated from the sea star organs by the extraction methods used. Furthermore, a muscle-relaxing secretion would seem superfluous, at least in the predation of *Asterias forbesi* upon *Mytilus edulis*, since it was shown above that this asteroid is capable of producing a pulling force which is transmitted to the valves of mussels by the anchoring action of the tube feet.

It may be questioned whether some species of pelecypods which are attacked by sea stars might not require stronger pulls to open than those that can be mustered by *Asterias*. Reese (1942) showed that 3750 grams could be withstood for several days by some *Venus* and *Ostrea;* Tamura (1929) reported that the Japanese oyster may sustain 15,000 grams pull for as long as five minutes; Galtsoff (1952) referred to the ability of oysters to withstand 6000 grams for several hours; Plateau (1884) computed *Ostrea's* "absolute resistance" (equal to the force required to open its shell one mm.) at 5026 grams, while Marceau (1909) reported that *Mytilus* could withstand a pull of 11.3 kg./sq. cm. of its adductor muscle tissue.

These impressive figures seem to preclude any possibility that sea stars pull open the shells of *Ostrea* and *Venus*. But, on closer examination, Plateau's "absolute resistance" appears outstandingly significant—if a force of 5026 grams can produce an opening of one mm. in *Ostrea*, might not a lesser pull be sufficient to open the shell 0.1 mm., the smallest measured gape through which sea stars' stomachs have been observed to penetrate? Many of the objections to the mechanical theory in the past have been based on the supposition that much larger gapes would be required (Reese proposed 7 mm. as a minimum), and the fact that such wide openings could be effected only by tremendously strong forces which a sea star could not be expected to exert. The experimental results described above have shown that *Asterias* is capable of producing pulling forces equivalent to 3100 grams. It seems likely that even greater forces could be demonstrated with adequate apparatus. Therefore, there is little reason to suppose that the usual bivalve prey of sea stars cannot be opened by the attached tube feet, at least enough for the insinuation of the stomach. According to this view, only very large and highly resistant molluscs would be immune to sea star predation. In fact, the larger, more resistant *Mytilus edulis* are seldom attacked successfully by sea stars. However, Feder (1955) reports that the larger *Mytilus californianus* are eaten by asteroids, but that entry into the shell is gained by way of the mussel's byssus "door" which is relatively wide in that species. By contrast, only one among the hundreds of east coast *Asterias* observed during this research was seen to have employed this approach. Feder also measured forces and shell openings which closely approximate the figures reported herein.

It must be emphasized that the observations made during this investigation do not support the popularly accepted notion that the process of predation is a "tug-of-war" in which the sea star becomes the victor by virtue of its persistence and greater endurance. The penetration is effected, as shown above, quite rapidly and as the result of a sudden overwhelming force, which is relaxed and re-applied at intervals until digestion of the soft parts of the bivalve has proceeded to the point where the adductor muscle is rendered ineffective.

The exact mechanism responsible for the pulling force has not been established. However, it is thought to reside in the musculature of the tube feet described in detail by Smith (1937, 1947). Once humped over the bivalve, the asteroid's body moves very little or not at all, but the tube feet are very active, protracting and retracting in such a way that they give the impression of operating in relays. Each tube foot's muscular tissue is ample to overcome the 29 grams of adhesiveness of the base (Paine, 1926). If this value is used as a criterion, then, it would appear that a sea star would need to employ less than one-fourth of all its tube feet simultaneously to produce pulls of over 5000 grams.

Summary

1. An investigation was made into the possibility that sea stars secrete a substance which is toxic or anesthetic for bivalves. Extracts prepared from the organs of feeding and non-feeding *Asterias forbesi* were introduced into the adductor muscle and the mantle cavity, or perfused over the beating heart, of *Mytilus edulis*. The effects of such solutions were, generally, identical to those produced by sea water or distilled water.

2. Sea stars were induced to feed upon specially prepared mussels, so that the forces which their tube feet exerted on the shells could be measured manometrically. The adductors of the mussels used in such experiments had been severed and replaced by steel springs or plastic cylinders which could not be affected by any alleged toxin. It was found that the tube feet did pull the valves apart and forces of over 3000 grams were recorded. It was observed also that a very minute opening between the valves (0.1 mm.) was sufficient to permit the insinuation of the asteroid stomach.

3. The common interpretation of the mechanical theory, which asserts that the sea star "fatigues" the mollusc, appears inaccurate in view of the findings of this research. There is evidence that the opening of the valves is a rapid process involving overwhelming, discontinuous forces, so that the predator may be considered to relax its pull upon the valves at intervals and to allow its stomach to be compressed between the valves until it pulls them apart again.

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STUDIES ON MARINE BRYOZOA. VIII. EXOCHELLA LONGIROSTRIS JULLIEN 1888

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The writer wishes to express deep gratitude to the National Science Foundation for research grants aiding this and other studies and to the Smithsonian Institution, U. S. N. M., for the loan of bryozoan specimens collected during the U. S. Navy's 1947–48 Antarctic Expedition by Comdr. David C. Nutt.

The purpose of this study is to report *Exochella longirostris* Jullien 1888 (order Cheilostomata, Family Exochellidae) from the Antarctic, to raise some questions about its synonymy and to add further morphological and ecological data to the limited information existing on this species.

Exochella longirostris Jullien 1888

(Figures 1 A–J)

Synonymy and distribution data:

1888. *Exochella longirostris.* Jullien pp. 55–56, Pl. 3, Figs. 1–4; Pl. 9, Fig. 2. From Ile Hoste, baie Orange, Canal du Beagle, Ile Gable, Tierra del Fuego. 19 meters.

1904. *E. longirostris.* Calvet p. 29. Magellan Straits, Punta Arenas.

1908. *E. longirostris.* Canu p. 300, Pl. VI, Fig. 13. From Post-Pampeen de Punta Borja, Puerto Militar, Bahia Blanca (Argentina).

1937. *E. longirostris.* Marcus pp. 82–83; Pl. 17, Fig. 43. Bay of Santos, Brazil; 20 meters.

1941. *E. longirostris.* Marcus p. 22; Fig. 16. Sta. Catharina, Parana; Guaratuba.

1949. *E. longirostris.* Marcus p. 1. South of Victoria, Espirito Santo, Lat. 20°33′S., Long. 40°14′W.; 35 meters.

1952. *E. longirostris.* Mawatari p. 265. Wakayama Prefecture, Shirahama and Tonda, Japan.

Some difficulty was encountered in the identification of this species because Jullien's original description was inadequate. Externally, the USNM specimens resemble those pictured by Jullien (1888) and Canu (1908) but these authors did not figure the internal aspect of the primary orifice, a very important diagnostic character. Levinsen (1909, p. 321; Pl. 17, Figs. 6a–c) beautifully and completely described an *Exochella longirostris* from Challenger Sta. 315, Falkland Islands. However, it is not at all certain that Levinsen and Jullien were describing the same species. Levinsen pictured a distinct lyrula on the proximal border of the primary

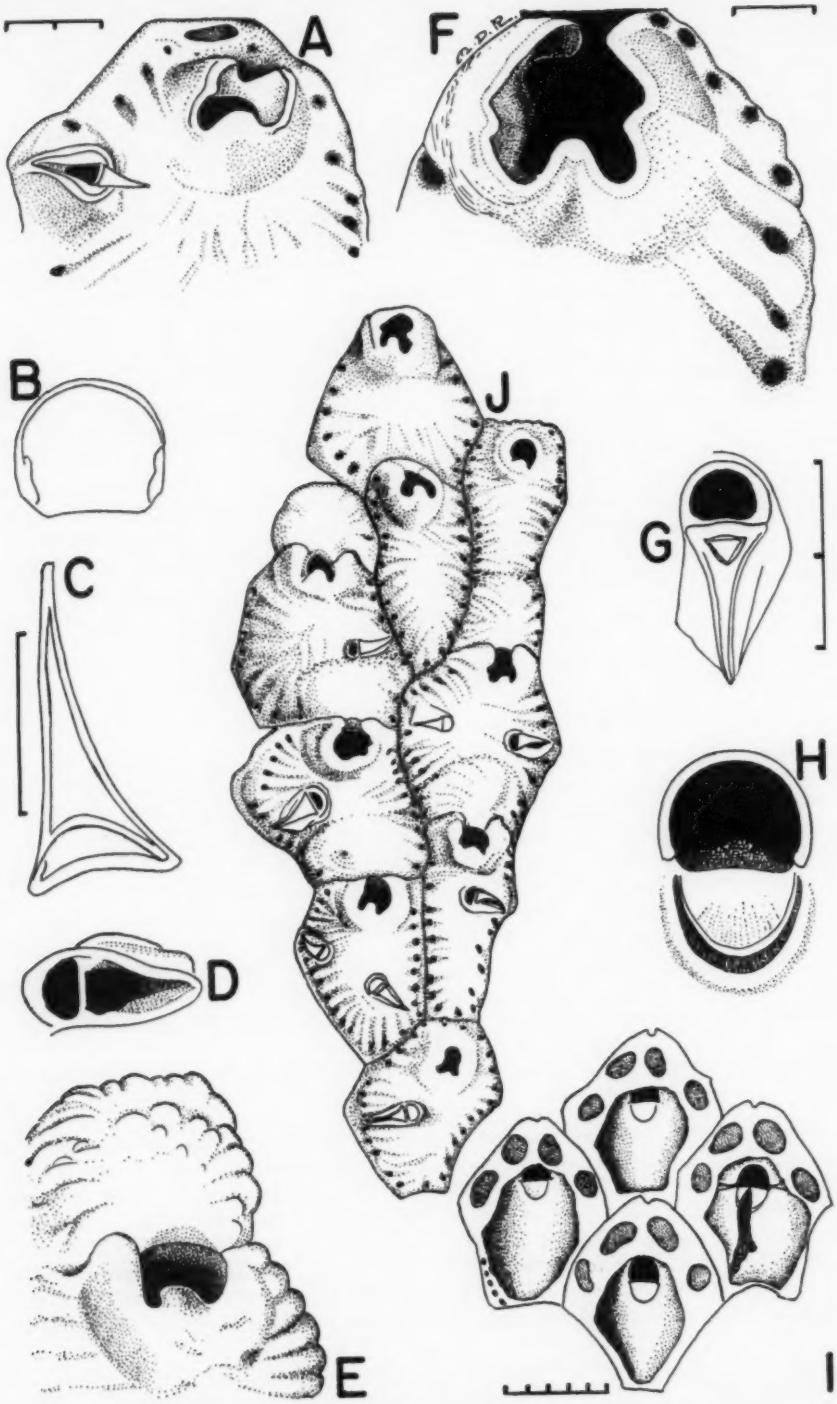

FIGURE 1, *A–J*.

orifice, while Jullien stated that the orifice is rounded and that its peristome is prolonged forward and backward. The USNM specimens do not show such a prominent structure so immediately within the primary orifice but do show the peristome thickened medially to simulate a lyrula a little in front of the primary orifice border. Waters's notations on this species (1889 *E. longirostris* and 1906 *Smittia longirostris*) are not precise enough for one to be able to determine if he actually had Jullien's species, so are not included in the present synonymy. Marcus' Bay of Santos specimens are considerably smaller in all parts (zooecia, apertures, ovicells, avicularia) than the USNM specimens, judging from the scale accompanying Marcus' Pl. 17, Fig. 43. His avicularia seem much thinner than those of the present specimens. Finally, the peristomial processes appear to be thinner and sharper than those of the USNM material. In spite of these differences, it is believed that these belong to the same species and that the differences are due to ecological and geographical factors, the USNM Antarctic specimens showing the sturdiest and largest specimens of this variable form.

Diagnosis: Zoarium encrusting, heavily calcified. Zooecial boundaries distinct. Convex frontal an areolate pleurocyst, somewhat ribbed in old zoids. Ovicells non-porous, covered over by the frontal of the next distal zoid. Avicularia adventitious, pointed, medium-sized, frontal, not peripheral nor over an areolar pore; one, two or none may occur on a zoid. Mandible long, triangular, sharply pointed. Peristome incomplete distally in ovicelled zoids. Raised peristome develops a mucro, sometimes medially thickened to simulate a lyrula. Peristomial sinus on each side of the mucro. Peristomial side walls raised, sometimes pressing inward. Primary orifice has a hemispherical vestibular arch. Immediate lyrula and cardelles absent.

All figures are drawn with the aid of a camera lucida.

FIGURE 1. *Exochella longirostris* Jullien 1888, from the Antarctic.

A. A zooecium at growing edge of colony. Thin young peristome still incomplete in back, with two sinuses and a lip-like mucro proximally. Mandible opened. One dietella (broad distal pore) shown at top. Drawn to the 0.2 mm. scale above.

B. Operculum, drawn to the Figure G scale.

C. Mandible, drawn to the 0.1 mm. scale at left.

D. Avicularium with membranous part burned off. Drawn to the Figure G scale.

E. An old ovicelled zooecium. The very thick peristome is worn away in front and at right. The projection simulating a lyrula is not a true lyrula but a thickened "core" of the mucro, a thickening of the proximal peristomial wall and characteristic of the most heavily calcified zooecia. Drawn to the Figure A scale.

F. Another young incomplete peristome. The mucro is more pointed and the side walls press in more acutely than in Figure A. Drawn to the 0.1 mm. scale above.

G. An avicularium with mandible in place. Membranous "back" area in black. Drawn to the 0.2 mm. scale at right.

H. The primary orifice in black, vestibular arch above, and compensation sac area below, as seen from inside the zooecium. Drawn to the Figure F scale.

I. Four zooecia seen from the attached basal side. Each has four dietellae (large heavily stippled distal pores). Five small areolar pores are on the lower corner of the left zoid whose basal wall has broken away there. The zooecium at right has the remains of the operculum and tentacular sheath suspended from the orifice. Compensation sac area also plainly visible in all. Drawn to the 0.5 mm. scale below.

J. Nine old zooecia. The upper three and the lowest one are non-ovicelled. The remaining five have non-porous ovicells more or less undistinguishable from the frontal of their distal zooecia. They can be recognized by the incomplete distal wall of the peristome. One, two or no avicularia may be present per zooecium. They are not areolar but are more central in location. Drawn to the Figure I scale.

Operculum forms three fourths of a circle, with proximal edge bevelled. Compensation sac about the size of the primary orifice. Three to five dietellae.

Measurements. The first figures are the minimum, the next the maximum and the last, in parentheses, the average of 10 readings for each structure (except for the avicularia whose averages are based on 30 readings). Length and width are abbreviated to L and W. Readings are in millimeters.

0.734–0.979	(0.888)	Zooecia L
0.605–0.922	(0.736)	Zooecia W
0.158–0.259	(0.204)	Avicularia L
0.072–0.130	(0.102)	Avicularia W
0.115–0.147	(0.131)	Primary orifice L
0.144–0.166	(0.155)	Primary orifice W
0.144–0.173	(0.153)	Secondary orifice L, including sinus
0.101–0.144	(0.124)	Secondary orifice L, exclusive of sinus
0.144–0.173	(0.153)	Secondary orifice W
0.302–0.360	(0.334)	Ovicell L
0.360–0.418	(0.382)	Ovicell W
0.137–0.158	(0.147)	Operculum L
0.130–0.173	(0.154)	Operculum W
0.128–0.151	(0.137)	Mandible L
0.058–0.073	(0.068)	Mandible W
0.115–0.158	(0.130)	Compensation sac area L
0.122–0.158	(0.143)	Compensation sac area W

Zoarium. The ivory-colored, heavily calcified zoarium is sturdy and sometimes extensive. A 25 × 36-mm. pebble had one surface completely encrusted by one colony. Colonies are unilaminate, forming a thick crust, usually numbering many zoids. Polypide remains present in some.

Zooecia. The hexagonal zooecia are distinct and sizable. Some are ovicelled, some not; some have avicularia, others do not. From the basal aspect (Fig. 1, I), the three distal walls are convex, the three proximal walls concave. The thick frontal is a granular to beaded pleurocyst. Ridges arise between its closely spaced elliptical areolar pores and continue part way up the frontal (Fig. 1, F, J). The compensation sac area is small and immediately below the orifice (Fig. 1, H, I). The basal, attached zooecial surface has 3 to 5, usually 4, large oval dietellae (Fig. 1, I).

Avicularia. One or two frontal avicularia occur on many of the zoids. Their orientation is variable on the solid part of the frontal. They are not oral nor areolar though some occur fairly close to the zooecial edge. Others are more central (Fig. 1, J). The small avicularial chamber tips the beak upward along a modest slope. The avicularia are always of the same type and of fairly uniform size. Their back area is hemispherical, the beak triangular and longer. The mandible is a narrow triangle, with the two long sides concave (Fig. 1, C, G), and edges reinforced. The USNM avicularia, though larger in actual measurements than those of Marcus' species, are smaller in proportion to the rest of the zooecial front than are Marcus' specimens.

Orifice. The orifice is not terminal but a slight distance short of that. Its

distal wall is not formed by the next distal zoid. The deeply set primary orifice is slightly more than hemispherical, with a handsome vestibular arch and a nearly straight proximal border (Fig. 1, H). The chitin-rimmed operculum has the same shape (Fig. 1, B). Lyrula and cardelles are absent in the primary orifice but the peristome immediately in front of the operculum simulates a lyrula. This appears to be at variance with Levinsen's figures which show a lyrula apparently right on the border of the primary aperture. Whether or not this is the condition of Jullien's original material is unknown. The secondary orifice shape is variable, depending on the degree of calcification, being sometimes trifoliate, sometimes horseshoe-shaped (Fig. 1, E, F, J). The distal peristome wall is entire in mature non-ovicelled zoids but interrupted by the ovicell in fertile ones. The peristome thickens considerably with age. Proximally the peristome develops a tab-like mucro (Figs. A, F) bordered on each side by a sinus. The mucro may thicken medially to such an extent inward that it could be easily mistaken for a lyrula (Fig. 1, E). Laterally, the peristomial wall may or may not pinch in (Fig. 1, F).

Ovicells. Young ovicells are salient, old ones heavily calcified and immersed. They are not porous but some are bordered laterally by a few areolar pores which do not penetrate the ovicell wall proper. The ovicell surface is granular to beaded, occasionally irregularly ridged (Fig. 1, E). No avicularia occur on the ovicells nor does the peristome encroach upon them but the frontal of the next distal zoid covers the ovicell front completely.

Distribution and ecology. This species' most northerly record (and the only one for the northern hemisphere) is that of Mawatari (1952) from Japan. All other previous records are from the southern hemisphere, ranging from 20°33′S. Lat. (Marcus, 1949, south of Victoria, Brazil) to about 55°40′S. Lat. (Jullien, 1888, Tierra del Fuego, Ile Hoste).

The USNM specimens appeared on a rock from Sta. 184 and on pebbles Nos. 2, 3, 4, 12, 13 and 16 from an unidentified Antarctic locality (Comdr. D. C. Nutt, U. S. Navy's 1947–48 Antarctic Expedition). Station 184 was at Marguerite Bay, Antarctica, location approximately 68°30′W. Long. and 68°30′S. Lat., bottom dredge haul, depth 85–100 fathoms, water temperature 30.2° F., Feb. 19, 1948. This represents the most southerly and deepest record for the species and the first time it was collected well within the Antarctic Circle. Some of the USNM colonies have grown over Foraminifera, incorporating their shells within the zooecial base. Sponge spicules are matted over one colony, calcareous worm tubes and occasional bryozoan zoids (of other species) are present on other colonies. However, most of the colony surface is free of extraneous growths. The Antarctic specimens appear to be much larger, thicker-walled and more sturdy than those from warmer localities. The present study specimens are on deposit at the U. S. Nat. Museum, Smithsonian Institution, Cat. Nos. 11325, 11326, 11327 and 11328.

Affinities. Exochella longirostris Jullien 1888 and a fossil species *E. grandis* Canu and Bassler (1935, p. 32, Pl. 9, Fig. 3) from the Tertiary Balcombian Beds of Muddy Creek, Victoria, Australia, appear to be closely related. The USNM specimens are similar in size and measurements to *E. grandis* but lack the prominent mural thread and the very conspicuous beading of the pleurocyst. In *E. grandis* the avicularia replace the areolar pores but in the USNM *E. longirostris* they generally do not and are less peripheral.

SUMMARY

1. The geographic range of *Exochella longirostris* is extended to the Antarctic.

2. The Antarctic specimens are sturdier, larger and thicker-walled than those of the same species from warmer waters and have avicularia which are a bit smaller proportionately, although larger in actual measurements.

3. Numerous measurements of various structures and zooecia are included, to show the range of variation for this species.

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MICROGEOGRAPHIC VARIATION AS THERMAL ACCLIMATION IN AN INTERTIDAL MOLLUSC

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A growing body of literature suggests that many poikilotherms are able to regulate, to a remarkable degree, their physiological activity rates. Regardless of the latitude over which certain species are distributed or the seasonal temperature change to which they are subjected, their physiological rates converge towards a mean value. To accomplish this relative constancy, northern populations and winter forms often have higher rates of activity, metabolism and development than southern populations and summer forms when measured at the same temperature. Animals thermally conditioned in the laboratory have also shown this compensatory phenomenon.

Ample documentation is provided in the comprehensive review of Bullock (1955), who also presents data showing temperature adaptation at the tissue, cellular and enzyme level, and in that of Prosser (1955), who summarizes evidence of compensatory adjustment to oxygen tension, osmotic pressure and drugs, as well as to temperature. Roberts (1952) and Dehnel (1955), who themselves have contributed studies of this problem, give additional references.

In 1953, Segal, Rao and James extended the known cases of intraspecific physiological differentiation with respect to temperature to include microgeographically separated individuals of the species. The heart rate in the limpet *Acmaea limatula* and water propulsion in the mussel *Mytilus californianus* were faster in samples from low intertidal levels than from high levels at any given temperature.

In the present study, an attempt has been made to corroborate and extend the initial findings on *A. limatula* and to ascertain whether the significant parameter of the difference in microhabitats is temperature. Besides heart rate, differences in gonad size and spawning readiness have been investigated. A major portion of this study constitutes an attempt to test the hypothesis that we are dealing with individual adaptations to habitat temperature.

Materials and Methods

Habitat

The aspidobranch gastropod *Acmaea limatula* Carpenter is a eurytopic intertidal species which at Palos Verdes, California (Lat. 33° 43′ N., Long. 118° 16′ W.) has a vertical distribution of approximately 1½ meters from a mid-tidal to a low-tidal level. Highest and lowest individuals may be separated by as much as 20 meters

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[2] I wish to thank Dr. T. H. Bullock for his encouragement and guidance throughout the course of this investigation.

of sloping, rocky beach; the nature of the beach prohibits interchange between higher and lower levels.

At each of four collecting sites (referred to in the text as sites 1, 2, 3, and 4) the low-level specimens were taken from below zero datum (mean lower low water: U. S. Coast and Geodetic Survey Tide Tables, Pacific Coast) where they are at the temperature of the surf but for a few hours each month. The higher level specimens were taken 1 to 1½ meters above zero datum where they are subject to exposure about 50% of the time.

Collection and care of animals

Animals were removed from the substrate with a thin spatula and transported wet to the laboratory in enamel or plastic trays. In the laboratory the animals were covered with fresh sea water, aerated, and refrigerated at temperatures approximating the average ocean temperature for that season (see Fig. 7). Each day the water was replaced with fresh sea water at the same temperature.

During the winter and spring months an attempt was made to approximate the natural exposure time for high-level individuals. The water was poured off and the animals allowed to warm to room temperature and stand for 5–6 daylight hours.

Heart exposure

No later than 24 hours after collection, the heart was exposed by cutting a hole in the shell to the left and slightly posterior to the shell apex with a fine toothed trephine. This tool was designed to take different sized cutting heads: 3 mm. in diameter for cutting small shells, 4½ mm. in diameter for cutting larger shells. A retractable pin in the center of the trephine prevented the cutting edge from wandering. The surface of the mantle exposed by the hole was flushed clean of shell particles with a fine stream of sea water.

Each animal was numbered with colored lacquer, then placed in a 10-inch finger bowl (15 to 20 individuals per bowl) which contained 1–1½ inches of sea water. The animals were returned to the refrigerator for one–two days to allow the gut to empty and permit recovery of the animals from any possible operative shock.

Recording procedure

The day heart beats were counted, two finger bowls were placed on a wire mesh platform two inches below the surface in a 15-gallon aquarium. Water circulated through the aquarium at a constant temperature $\pm$ 0.2° C.

Initially, the water bath was at the refrigerator temperature and was then gradually lowered to 4°, 7°, or 9° C. Two to four hours were allowed for the animals to reach the lower temperatures. Temperatures were raised by increments of 5° C. to a maximum of 29° C. Animals were allowed two hours to reach each temperature.

Using a stop-watch, the number of seconds required for 10 heart beats was counted by eye. At lower temperatures a reading was taken of each animal in the group and this was repeated a second and third time. At higher temperatures, because of the greater possibility of error due to the increased heart rate, the read-

ings were taken 5 times. Plotted points (see Figs. 1, 3, and 4), are the average of these data converted to beats per minute.

The basic measurement used in this study is stable and reliable. A few minutes after the operation, which does not break the mantle, the heart rate settles to a value which is consistent over many hours and even days. Those animals showing excessive locomotor activity or irregularity of heart activity were discarded.

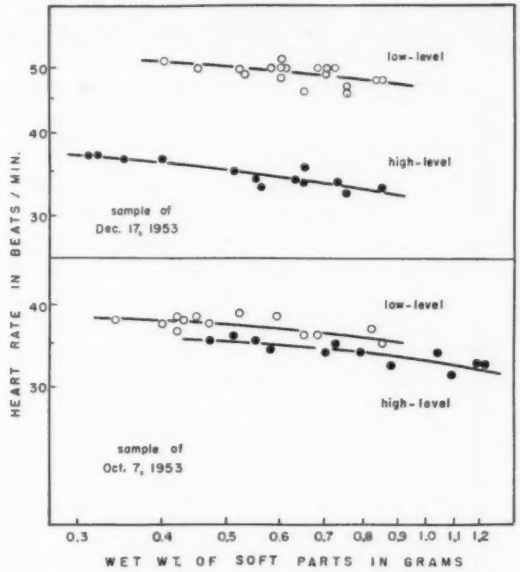


FIGURE 1. Relation between heart rate and wet weight of soft parts at 14° C. for vertically separated *A. limatula* from Palos Verdes. Points are averages of 3 to 5 readings of the number of seconds per 10 beats for one individual. The December collection shows a near maximum excursion of the difference in heart rate between samples. The October collection shows the minimum difference. Co-ordinates are logarithmic; equal percentage deviation is, therefore, shown by equal spatial spread from the hand-drawn regression lines anywhere on the graph. All curves are eye-fitted.

RESULTS

Effect of Intertidal Height on Heart Rate

When the animals' heart rates are measured at temperatures from 4°–29° C., it is found that the lower in the range of distribution an animal lives the faster is its heart rate. A comparison is made of the heart rates of "highs" and "lows" and the near maximum and the minimum difference in rate between samples are illustrated in Figure 1. At lower temperatures the absolute difference in heart frequency between "highs" and "lows" is less than it is at higher temperatures.

When heart rate is plotted against temperature, it can be seen that the curve for a low-level animal is above that for an equal weight individual from high-level

(Fig. 2). Since heart frequency will be shown to vary with habitat and season, rates at successive collections could not be combined. The pair of rate/temperature curves in Figure 2 have been selected from the many pairs calculated because they show the response both over the greatest range of temperatures and the largest number of temperature points.

When both groups are tested at any temperature, within the physiological ranges of temperatures of the species, heart rates of low-level animals are as much as 30 to 40% faster than those of equal weight animals from high-level. Heart rates of equal value for "highs" and "lows" are obtained when the high-level individuals are

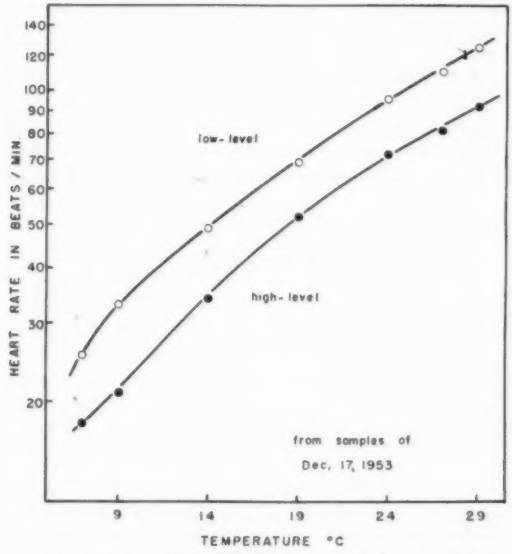


FIGURE 2. Heart rate as a function of temperature for equal weight (0.6 gm.) *A. limatula* from December collection of high- and low-level samples. Points represent intersection of perpendicular, erected at 0.6 gm., with weight regression curves at each temperature.

measured at temperatures 1°–5½° C. above that of low-level individuals. In February, for example (not graphed), a low-level individual of average weight (0.6 gm.) has a heart rate of 53 beats per minute at 14° C. An average high-level individual of equal weight shows the same rate when measured at 19.5° C.

In addition to the difference in position of the rate-temperature curves, there appears to be a reliable difference in the slopes as measured by the Q_{10}. Between 9° and 19° C., perhaps even above 19° C., the Q_{10} of the heart rate is consistently lower for low-level animals during the winter and spring months (Table I).

It would be of value to be able to state conclusively whether high-level individuals of the species are living at a warmer temperature than low-level individuals, at least within the local coastal area. An attempt will be made to establish this point.

High-level individuals are submerged approximately 50% of the time. During the hours of exposure these individuals are subjected to air temperatures which fluctuate about the prevailing water temperatures. A series of readings (taken with a thermistor probe, one mm. in diameter, inserted under the foot of limpets in place in the field) on a sunny day in late October show that body temperatures

TABLE I

Q_{10} values of the heart rate for 0.6 gm. high- and low-level A. limatula over the temperature ranges indicated. Values calculated for equal weight animals on the weight regression curves at each collection period

Date	Temp. ° C.	Q_{10}	
		High-level	Low-level
7/27/53	9–14	3.40	3.14
	14–19	2.56	2.53
	19–24	1.99	1.88
10/7/53	9–14	3.22	3.10
	14–19	2.38	2.89
	19–24	1.73	1.77
12/17/53	9–14	2.62	2.30
	14–19	2.34	1.98
	19–24	2.02	1.97
1/4/54	9–14	2.70	2.49
	14–19	2.18	2.16
	19–24	1.80	1.62
2/1/54	9–14	2.57	2.34
	14–19	2.54	1.97
	19–24	1.77	1.80
2/15/54	9–14	2.67	2.54
	14–19	2.38	2.05
	19–24	1.83	1.95
4/6/54	9–14	2.74	2.37
	14–19	2.02	1.78
	19–24	1.59	1.73
5/8/54	9–14	2.51	2.37
	14–19	2.11	2.02
	19–24	1.76	1.65

reached a high of 30° C. in the sun and 21.5° C. in the shade. Body temperatures may have reached higher values since the animals were exposed for an additional two hours. Over a 20-year period (U. S. Weather Bureau, personal communication) 40 to 50% of the days, from October through April, have been sunny at the local beaches. From November through April the surface water temperature averages slightly over 14.5° C.

Reference to Figure 8 shows that the annual range of the inshore surface water temperatures averages approximately 7° C. Air temperatures, taken a few feet above ground, show daily, and therefore monthly, fluctuations exceeding the yearly temperature range of the inshore waters. Of course, air temperatures a few feet above ground give only a rough directional estimate of microhabitat temperatures one cm. above ground. Over a period of 1½ years, dry bulb recordings have shown the microhabitat temperatures to be consistently higher than prevailing air temperatures.

Minimum air temperatures falling below ocean temperatures are encountered primarily from late spring to early autumn when low tides generally expose high-level animals during late evening and early morning hours. During the part of this period when the ocean temperature is above 17° C., the difference in heart rate between high- and low-level animals is at a minimum. The critical exposure occurs during winter and early spring in the late morning and afternoon hours. During this period the difference in heart rate is maximal.

It is of considerable interest, in this regard, that high-level animals show lower Q_{10}'s of the heart rate between 7° and 9° C. than low-level animals (low-level = 3.55, 3.66; high-level = 2.00, 2.15—two experimental recordings). It suggests that low-level animals are approaching cold depression at a higher temperature than are the high-level animals. The physiological temperature range is therefore believed to be wider for the relatively warm adapted high-level animals.

Influence of Certain Variables on Heart Rate

Body size. Among the numerous factors bearing influence upon physiological rate functions, size has been found to contribute to the variation in heart rate in the species under investigation. Size has been measured by the wet weight of soft parts. Within the weight range of 0.3 to 1.2 gm., larger animals show consistently slower rates at all temperatures from 4° to 29° C.

The regression of rate with weight is without apparent systematic variation over the year and is not significantly different between "highs" and "lows" at 14° C. ($P = .35$ for the difference between mean regression coefficients of 11 high-level and 12 low-level samples). Ten of the 23 samples show no reliable difference in regression from 9°–24° C. The remaining 13 samples have larger negative b values on either side of 14° and 19° C. Hence, no single expression is available to describe fully the effect of weight on heart rate in this species.

Within the weight range of 0.4 to 1.0 gm., the regression is usually linear when plotted on logarithmic coordinates and varies from -0.043 to -0.172. However, it is non-linear on either side of this weight range. It is as if we were plotting only a segment of a large parabola (see Figs. 1 and 3).

Both the factors of size and individual variation contribute to the scatter about each regression line. Size, however, is the major factor producing the scatter. Since weight and rate are inversely related, rate differences are meaningful only with essentially equal weight animals.

Sex. Sexes in *A. limatula*, as in all species of the genus, are separate, but three rather than two sexual states are present. To the conventional male and female is added the condition of indeterminacy. The latter is simply the post-spawning phase of the male or female in which gametes are absent (see section on gonad size).

It has not been possible to find a differential effect upon the heart rate that can be attributed to any of the sexual states excluding the pre-spawning animals heavy with gonads (see below).

Gonad size. An analysis of possible reproductive patterns will be presented in a following section. It is obvious from the data that the size and condition of the gonads vary over the year and between vertically separated individuals. Size of the gonad as such has a negligible effect on the heart rate; the condition of the gonads is, however, of importance.

On occasion when as many as 50% of a sample were possessed of insignificant gonads or were of indeterminate sex, and the remainder showed gonads weighing up to 20% of the body weight, the heart frequency of all animals fell within the scatter of either group. On the other hand, pre-spawning buildup of gonadal tissue, regardless of the size attained, rendered the heart beat erratic and not reliably measureable under our conditions. This was found in July for both high- and low-level individuals. In the latter part of the same month, when comparing heart rates of samples having approximately equal gonads by weight, high-level individuals showed slower rates consistent with the difference between groups throughout the year.

Diurnal rhythms. Two groups of 5 animals each were maintained at 14° C. and the heart beat counted at one-hour intervals over a 20-hour period. The animals were under constant illumination. Under these conditions, the presence of a day-night rhythm could not be demonstrated among either the high- or low-level individuals.

Effect of Transplantation on Heart Rate

Twenty-nine days. During March, 1953, 42 high-level and 42 low-level limpets were reciprocally transposed at site 1. Fifty control specimens from each level were handled in like manner but returned to their natural positions (see section on behavioral response). The numbers of recoveries are presented below.

Individuals transposed from low-level to high	52%
Individuals transposed from high-level to low	57%
Control individuals from high-level	84%
Control individuals from low-level	60%

Twenty-nine days after reciprocal transplantation, the heart rate, when measured from 4°–29° C., appeared to have undergone a complete reversal. Figure 3 shows the heart rate response of the transplants and controls at three selected temperatures. Heart rates of high-level individuals introduced into the low-level tide pool show a remarkable degree of overlap with those of the tide pool controls. An exception may be noted in the case of three individuals above 1.00 gm. in weight. However, since the rates for these animals fall within the variation of all transplants about the regression line, no significance has been attached to them.

Tide pool individuals transposed to high-level show heart rates close to but consistently faster than those of the high-level controls. Since animals transposed to the high-level position moved from their sites of placement to more protected positions (see below), the migrants have acclimated to a temperature somewhat lower than the "living" temperature of high-level controls.

Fourteen days. During January, 1954, a second reciprocal transplantation was

performed at site 4. Recovery was exceptionally poor. Of 50 high-level individuals transposed to low-level and 50 low-level controls, all but 2 and 5, respectively, were lost. Of the same number of low-level individuals transposed to high-level, 20% were recovered as compared with 50% of the high-level controls. Heart rates of low-level controls at the end of the experimental period and high-level transplants to low-level were therefore not available for comparison in significant numbers.

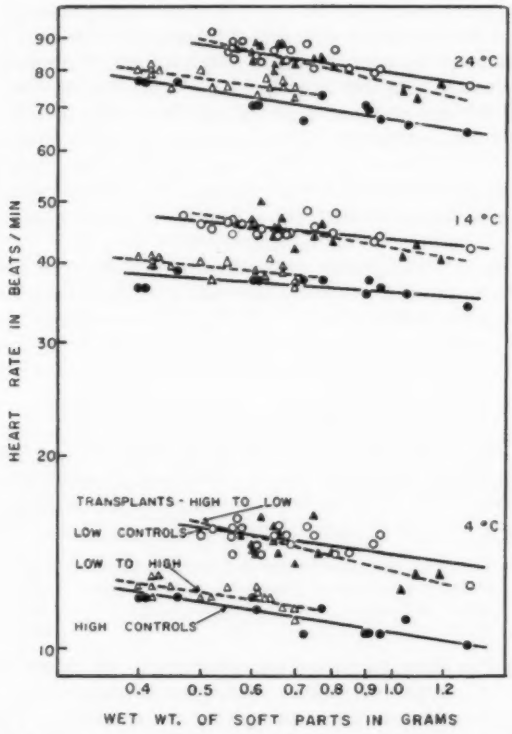


FIGURE 3. Relation between heart rate and wet weight of soft parts of reciprocal transplants and controls 29 days after transplantation. Each point represents one individual.

Comparing animals of equal weight, it is evident that the transplants to high-level have a lower heart frequency than initial low-level controls and a higher frequency than high-level controls at any temperature from 9°–24° C. (Fig. 4). The degree of acclimation of the transplants cannot be stated in equivalent °C., since the thermal history in the field cannot be given in simple terms. We only know that partial acclimation has occurred.

If we calculate the change in heart frequency of the transplants as a percentage of the difference in the frequencies of the low- and high-level controls, at the begin-

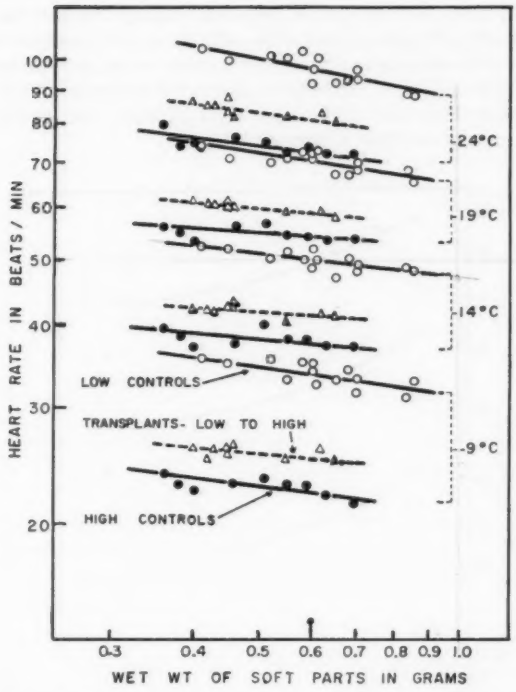


FIGURE 4. Relation between heart rate and wet weight of soft parts for second experimental transplantation. Duration of experiment 14 days. Each point represents one individual. Low-level controls measured at start of experimental period; high-level controls measured at end.

TABLE II

Calculation of per cent acclimation after transplantation from low- to high-level. Heart rate values are from equal weight (0.6 gm.) high- and low-level controls at start of experiment and high controls and transplants to high-level after 14 days. Rate values are taken from Figure 4. Per cent acclimation of transplants calculated from the difference in rate of low- and high-level controls at beginning and end of experiment. Performed in January 1954.

Temp. ° C.	Mean heart rates of average 0.6 gm. animals in beats/min.				% acclimation
	High controls 12/17/54	Low controls 12/17/54	Transpl. to high 1/4/54	High controls 1/4/54	
9	21.0	33.0	25.0	22.5	76.0
14	34.0	49.0	41.0	37.0	66.5
19	52.0	69.0	59.0	54.0	66.5
24	73.0	97.0	81.0	72.5	65.5
29	92.0	126.0	106.0	95.0	64.5

ning and end of the experimental period, we can roughly compare the response after 14 days with that after 29 days (Table II). At the 5 temperatures shown, an average of 68% of the difference in heart frequency between the groups has been achieved by the transplants in 14 days. However, since the transplants are acclimating to a temperature lower than that of the high-level habitat (see section on behavioral response), 68% may be too low a figure.

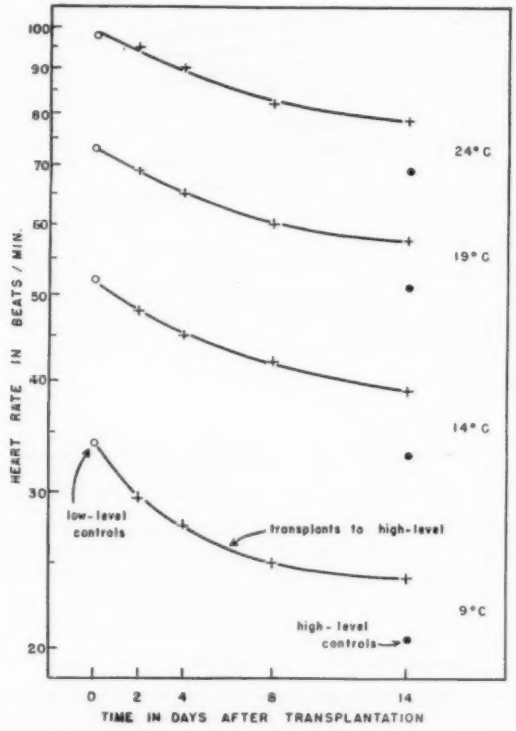


FIGURE 5. Acclimation of heart rate in low-level transplants to high-level 2, 4, 8, and 14 days after transplantation. Points represent equal weight (0.6 gm.) animals taken from the weight regression curve of each sample. Low-level controls measured at beginning of experiment, high-level controls at end and low-level transplants to high-level at days indicated.

It is of interest to notice that there is a proportionately greater change in heart frequency of the transplants at 9° C. than at any of the higher temperatures up to 29° C. This differential response may have as a basis the difference in slope of the rate/temperature curves of high- and low-level controls. The Q_{10}'s of the heart rate show the low-level or cold acclimated group to be less sensitive to temperature change at lower temperatures, *i.e.*, the curve is flatter between 9° and 14° C. An average 0.6-gm. animal from low-level has a Q_{10} of 2.33, while that of equal weight

TABLE III

Calculation of per cent acclimation 14 days after transplantation from low- to high-level and calculation of Q_{10} at several temperatures, 2, 4, 8, and 14 days after transplantation. Heart rates obtained as in Table II. Per cent acclimation calculated as in Table II. Performed February 1954.

Temp. ° C.	Heart rates of average 0.6 gm. animals in beats/min.							Acclimation after 14 days %
	High cont. 2/1/54	Low cont. 2/1/54	Trans. to high. Days after trans.				High cont. 2/15/54	
			2	4	8	14		
9	20.0	34.0	29.5	27.5	25.0	24.0	20.5	74.0
14	32.0	52.0	48.0	45.0	42.0	39.0	33.0	68.5
19	51.0	73.0	69.0	65.0	60.0	57.5	51.0	70.5
24	67.0	98.0	95.0	90.0	82.0	78.0	69.0	67.5
	Q_{10} values from these figures							
9–14	2.56	2.34	2.65	2.68	2.80	2.64	2.67	
14–19	2.54	1.97	2.07	2.08	2.04	2.17	2.38	
19–24	1.77	1.80	1.90	1.92	1.87	1.86	1.83	

high-level and transplant to high-level is 2.70 and 2.69, respectively, after 14 days. The difference in Q_{10} decreases with increasing temperature. The change in slope of the rate/temperature curve requires a proportionately greater change in heart frequency at lower temperatures.

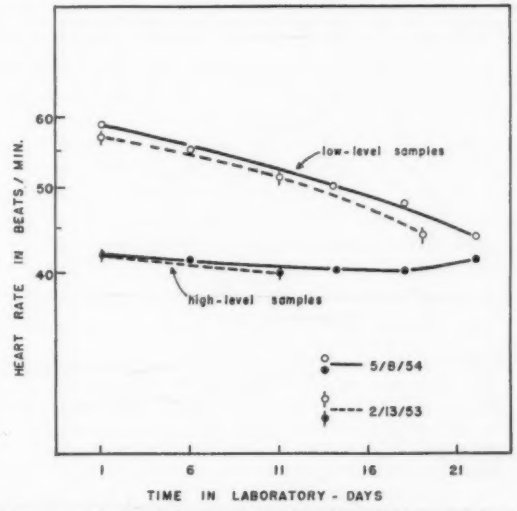
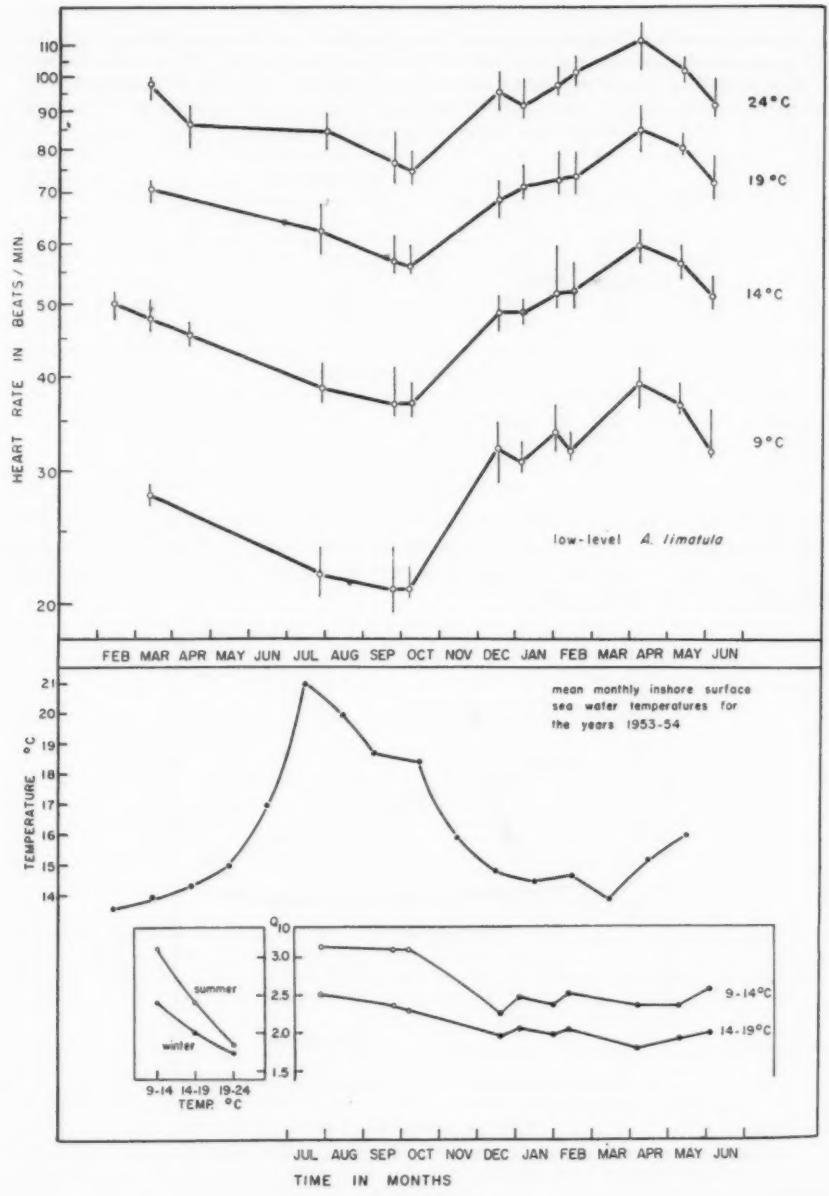


FIGURE 6. Heart rate during laboratory acclimation. High- and low-level samples kept cool (14° C.) and without food. Samples taken on days indicated; heart rate recorded at acclimation temperature (14° C.). Points represent equal weight (0.5 gm.) "average" animals taken from weight regression curves.


HEART RATE IN BEATS / MIN.
110
100
90
80
70
60
50
40
30
20
24°C
19°C
14°C
9°C
low-level A. limatula
FEB MAR APR MAY JUN JUL AUG SEP OCT NOV DEC JAN FEB MAR APR MAY JUN
TEMPERATURE °C
21
20
19
18
17
16
15
14
mean monthly inshore surface sea water temperatures for the years 1953-54
Q10
3.0
2.5
2.0
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summer
winter
9-14 14-19 19-24
TEMP °C
9-14°C
14-19°C
JUL AUG SEP OCT NOV DEC JAN FEB MAR APR MAY JUN
TIME IN MONTHS

FIGURE 7.

Fourteen-days: time course. In early February, 1954, 100 low-level limpets were moved to high-level at site 4. A total of 60% were recovered. Of 50 low- and 50 high-level controls, 50% and 60%, respectively, were recovered. Transplants were collected 2, 4, 8, and 14 days after transplantation.

Within two days a decrease in the heart rate of transposed individuals is noticeable. The further decrease in heart rate is non-linear plotted semilogarithmically, declining most rapidly in the first 8 days (Fig. 5).

Roughly 70% of the difference in heart rate was achieved by 14 days (Table III). This figure is arrived at by using the high-level control values (at 14 days) as a criterion of complete acclimation. However, for the reason given above (see second experimental transplantation), 70% is too low a figure.

The response of the transplants to short term temperature steps is remarkably similar in both this and the previous experiment. Not only is there a proportionately greater decrease in heart frequency at 9° than at 24° C. after 14 days, but the differential response is apparent after two days. Table III shows the calculated Q_{10}'s of the heart rate for equal weight controls and transplants. The change in slope at the lower end of the rate/temperature curve is conspicuous within two days.

Behavioral Response to Transplantation

On all three occasions, when low-level limpets were transposed to the level of high-water individuals, it was observed that the transplants moved from the site of placement.

In the first group of low- to high-level transplants, recovered on April 11, 1953, 29 days after transplantation, all surviving animals were found buried beneath a shell and gravel deposit at depths to 6 inches. High-level controls were recovered from the basalt outcroppings where they were naturally located, whereas the transplants, originally placed in close proximity to the controls, had migrated vertically downward from 1–6 inches out of the direct sunlight and into the damp deposit. A return to their old level is considered to be impossible on the boulder strewn beach.

The identical behavioral response was elicited from transplants in subsequent experiments. All surviving animals were found to have moved either vertically downward or horizontally under overhanging rocks, in both cases into cooler and damper regions. During the closely watched experiment, migratory movements were found to be complete two days after placement. High-level individuals, again, remained where they had been placed, in some cases in the identical spot.

The behavioral response to transplantation has bearing upon the heart frequency relations and is discussed in connection with those measurements.

FIGURE 7. Heart rate as a function of season for low-level *A. limatula*. Upper four curves —horizontal lines connect points for equal weight (0.6 gm.) animals taken from weight regression curves. Vertical lines denote total variation around regression lines within the weight range of 0.4–0.8 gm. The weight selected for comparison is from the middle of the usual range of weight at any collection period and from the comparable linear segment of the regression curves. Fifth curve—mean monthly inshore surface water temperatures in °C. from Redondo Beach, California (area adjacent to northernmost collection site) for the years 1953–1954. Points are monthly means calculated from the values of four daily recordings made between 9:00 A.M. and 6:00 P.M. Lower two curves—Q_{10} values calculated from the data above (upper 4 curves) for the temperature intervals shown. Inset—Q_{10} plotted against temperature to compare summer and winter averages.

Laboratory Studies

Additional, though indirect, laboratory evidence substantiates the field studies on acclimation. High- and low-level *A. limatula* were maintained at 14° C. in the laboratory without food for periods up to 22 days. Five to 10 individuals were withdrawn, one day after collection and at intervals thereafter, and the heart beat counted at 14° C. The heart rate of an average weight low-level animal, selected from the weight regression curves, decreased 25% of the initial rate after 22 days. A similarly obtained equal weight animal from high-level showed no appreciable decrease in rate in the same number of days (Fig. 6).

At the time of collection, low-level animals were living in the field at approximately the temperature of the experiment. The decrease in heart rate is presumed to be due to starvation uncomplicated by a tendency to acclimate. High-level animals, on the other hand, were living in the laboratory at a temperature lower than that in the field. At 14° C. two opposing forces are at work on these animals: starvation tending to decrease the heart rate and acclimation to a lower temperature tending to increase it.

Effect of Season on Heart Rate

Low-level population. Low-level *A. limatula*, collected during the winter and spring, have faster heart rates at all temperatures from 4° to 29° C. than animals of equal weight collected in summer (Fig. 7). The seasonal trend in heart frequency is in good agreement with the change in the mean monthly surface water temperature. In general, there is an inverse relationship such that an increase in temperature is followed by a decrease in heart rate which increases again with waning temperatures. The difference in winter and spring recordings of 1953 and 1954 has a basis in the habitat differences of the samples taken during those periods. Animals were collected from different sites during the comparable periods of each year. Low-level animals collected in 1953 were apparently slightly more subject to warming than were those animals collected in 1954.

The mean absolute change in rate (from winter to summer) at 24° C. is greater than at lower temperatures but the proportionate change at 9° C. exceeds that at higher temperatures. At 9° C. there is a maximum 46% change in rate as compared with 29% at 24° C.

Temperature sensitivity of the heart rate (as measured by the Q_{10}) similarly changes with season (see Fig. 7 and Table I). Winter and spring animals show lower Q_{10}'s and thus decreased sensitivity to temperature change between 9° and 24° C. With increasing temperature the difference in Q_{10} between winter and summer animals decreases. There is no appreciable difference if we compare Q_{10}'s at temperatures at which winter and summer animals show equal rates (at 14° C. or above for winter animals).

High-level population. When the seasonal change in heart rate of high- level forms is examined, the picture is less clear than that obtained for low-level forms (Fig. 8). There is a suggestion of the inverse relationship with seasonal temperature change, but the range in heart frequency is smaller and a response to short term temperature fluctuations, of the order of several days to a week, is evident (see February, 1954, Fig. 8). Although high-level animals were always chosen from the upper extreme of the intertidal range of the species, the possibility of habitat differences between samples cannot be ruled out.

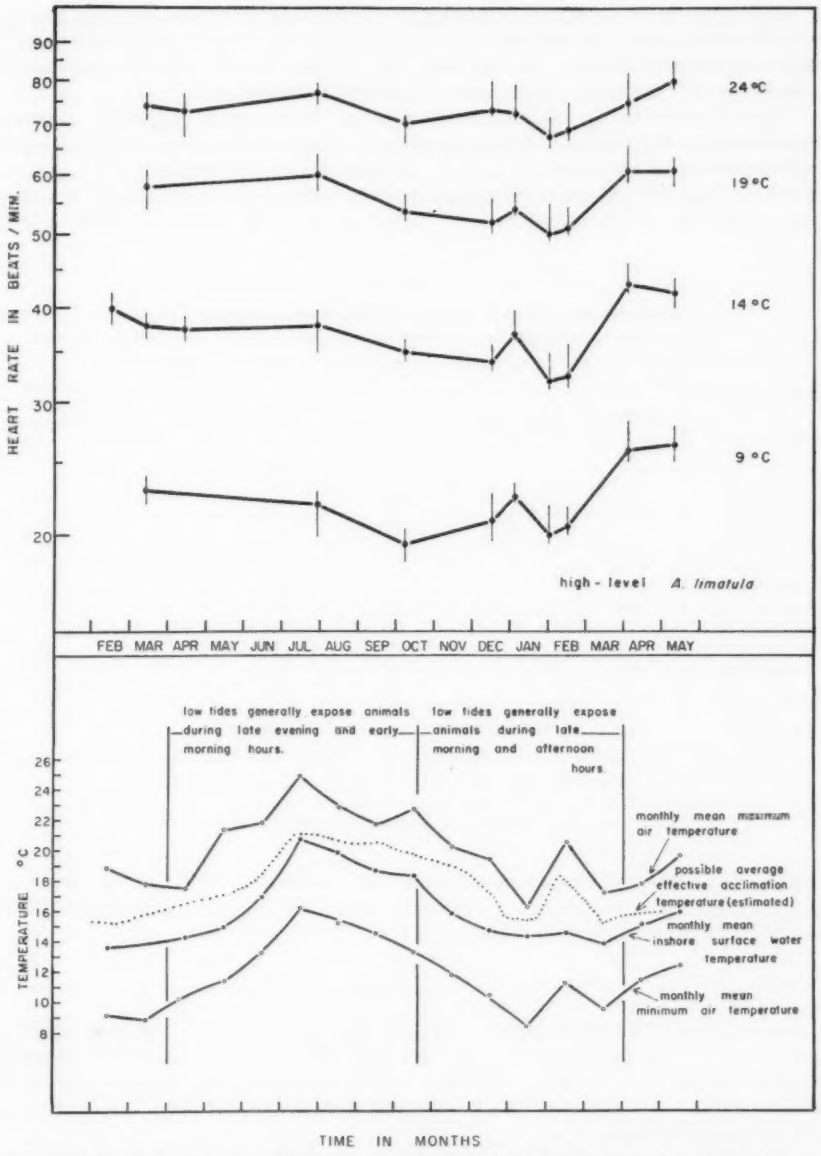

FIGURE 8. Heart rate as a function of season for high-level *A. limatula*. Parameters of upper 4 curves are the same as in Figure 7. Lower series of curves show (1) mean maximum and minimum monthly air temperatures (—○—) taken 4 times daily at Santa Montica, California; (2) monthly mean inshore surface water temperatures (—●—) as in Figure 7 but on reduced scale; and (3) possible average effective acclimation temperature (estimated) based on the heart rate response (· · · · ·).

High-level animals begin pre-spawning buildup of gonadal tissue in July and appear to maintain ripe gonads through October (see section on gonad size). The rather rapid heart rates in late July and possibly also in early October may be due to an increased metabolic activity associated with gametogenesis.

Table I shows the Q_{10} values calculated from the curves in Figure 8. Samples taken during "characteristic" winter months (January, April) show decreased temperature sensitivity (lower Q_{10}'s) than samples taken in "characteristic" summer months (August). However there is a marked lack of consistency in the temperature sensitivity of the heart rates of high-level animals. Samples taken during

TABLE IV

Gonad size as a function of season in high- and low-level A. limatula

Date of collection	Average gonad size in % wet weight of soft parts					
	High level	Gonads turgid	No. of specimens	Low level	Gonads turgid	No. of specimens
2/13/53	8.6		20	29.3		26
3/13/53	7.6		14	16.3		12
4/11/53	0.0		22	16.1		21
7/12/53	—	X	18	—	X	18
7/27/53	21.8	X	14	20.2*		7
9/25/53	28.1†	X	6	—**		12
10/ 7/53	29.4		12	17.1		15
12/17/53	6.6		13	21.7		19
1/ 4/54	7.6		20	31.1		12
2/ 1/54	0.0		16	20.4		15
2/15/54	0.0		12	15.9		10
4/ 6/54	0.0		14	17.0		18
5/ 8/54	0.0		8	15.6		11
6/12/54	15.6		14	16.1		12

* Six additional animals spawned in field—weights not taken.
** All animals spawned in field—weights not taken.
† Five additional animals spawned in laboratory—weights not taken.

months of unseasonal temperature fluctuations (February, 1954) do not show the Q_{10} associated with adaptation of the heart rate to a warmer temperature, although the rates themselves have adapted.

Gonad Size

In relation to intertidal height. Gonad size, as per cent of the wet weight of soft parts, has not been found to vary systematically with the size of the organism over the range of 0.3–1.2 gm. Therefore, the mean gonad weight, as per cent of the body weight, of the animals of each collection period was calculated. These values are presented in Table IV.

Excluding the period roughly from early June to mid-October, low-level *A. limatula* appeared to maintain a larger gonad than high-level forms of the species. Mean gonad weight of low-level individuals did not fall below 15% of the body weight dur-

ing the 1½-year period of observation. High-level individuals, from approximately November until June, possessed either small or negligible amounts of gonadal tissue with as many as 60% showing indeterminate sex. Indeterminate sex among low-level individuals was observed during the month of September after spawning occurred in the field.

In relation to season. Natural spawning among high- and low-level individuals does not appear to occur at similar times. Turgidity of the gonad (regardless of size) and deformation of the female gametes have been used to indicate the presence

TABLE V

Effect of transplantation on size of gonad in A. limatula

Date of collection	Average gonad size in % wet weight of soft parts							
	Controls				Transplants			
	High level	No.	Low level	No.	Low to high	No.	High to low	No.
Experiment I—29 days								
3/13/53	7.6	14	16.3	12				
4/11/53	0.0	22	16.1	21	0.0	18	15.9	20
Experiment II—14 days								
12/17/53	6.6	13	21.7	19				
1/ 4/54	7.6	20	31.1	13	21.9	10		
Experiment III—at intervals to 14 days								
2/ 1/54	0.0	16	20.4	15				
2/ 3/54					19.9	9		
2/ 5/54					21.4	9		
2/ 9/54					21.2*	4		
2/15/54	0.0	12	15.9	10	7.3**	4		

* Six other animals spawned in laboratory.
** Six other animals without weighable gonads at time of collection.

of pre-spawning ripeness—in agreement with Fritchman (1953). In July, samples from both high- and low-level revealed this condition (see Table IV). Later in the same month, partial or complete spawning of 50% of the low-level sample had occurred. Spawning of the entire low-level population is assumed to have taken place before late September.

High-level individuals, on the other hand, showed a persistent turgidity of the gonads throughout late September in the field, although under maintained temperature (17°) in the laboratory, 50% of the September sample spawned. In early October, weight of the gonads of high-level forms had not significantly changed, but turgidity was no longer apparent. By December, 50% of the high-level sample

was devoid of weighable gonadal tissue; by February, 100% of the animals showed this condition and remained so until the following June. After September, spawning among the low-level population was apparently at an end; the gonads then returned to approximately the average weight for non-spawning months. However, a second buildup of the gonads occurred in midwinter (January and February of two consecutive years) which, though of substantial weight, did not show the characteristic pre-spawning turgidity.

Effect of transplantation. Gonads of transposed animals were examined subsequent to recording of the heart rate. Data are available from three experimental transplantations and have been summarized in Table V.

1. After 29 days, in the spring, a complete reversal of the gonad size was obtained. High-level transplants to low-level developed gonads whose average size was not significantly different from that of the low-level controls.

2. During January, 1954, the second gonadal buildup occurred among low-level individuals. While the average gonadal weight of low-level controls increased by aproximately 50% in 14 days, that of the transplants from low- to high-level did not increase; rather they averaged the same size as before transplantation.

3. During February, 1954, animals were collected 2, 4, 8, and 14 days after transplantation from low- to high-level. No change was observed in the size of the gonads up to 8 days after transplantation although 60% of the individuals from the eighth day of collection spawned in the laboratory (19° C.). At the end of 14 days, 60% of the transplanted individuals possessed negligible gonads and the weights of the remaining 40% averaged less than one-half that of the low-level controls on the same date.

Discussion

Microgeographic variation. The preceding data show that the microgeographic intertidal distribution of a gastropod, *Acmaea limatula*, is reflected in certain physiological and morphological differences (see Segal, Rao and James, 1953, preliminary report). The differences found in relation to shell and body size have been reported previously (Segal, 1956). High intertidal *A. limatula* are found about one meter above zero datum; low intertidal *A. limatula* are found at zero datum and below. High-level forms show a slower heart rate than low-level forms when both are measured at any given temperature from 7° to 29° C. Comparisons show that high intertidal animals are exposed about 50% of the time and are subjected to air temperatures which rise above and fall below that of the ocean. Low intertidal animals are submerged over 90% of the time and live essentially at the temperature of the ocean.

We do not have the complete curve of temperature against time for the high-level animals; therefore, we do not know how the temperatures they are acclimated to are related to the temperature fluctuations they are subjected to. We only know that these animals respond as if they are living at a higher temperature than that of the ocean. In this regard, Kirberger (1953) maintained an annelid, *Lumbriculus variegatus*, for 12 hours alternately at 16° and 23° C. for 8 to 14 days. She compared the O_2 consumption of these animals with that of two groups kept solely at 16° and at 23° C. for the same period of time. Those kept at the alternating tempera-

tures averaged the fluctuations and responded as if they were adapted to 19° C. Animals maintained at the constant temperatures showed the typical compensatory response, *i.e.*, those animals from 16° C. consumed more O_2 than those from 23° C. when measured at the same temperatures.

Numerous studies have shown similar physiological differences to exist between macrogeographically distributed populations of a species. The question has been raised as to whether these animals are, in fact, members of the same species. Such latitudinal studies where the physiological differences are clearly correlated with habitat temperatures (Mayer, 1914; Spärck, 1936; Fox and Wingfield, 1937; Fox, 1939; Roberts, 1952; Dehnel, 1955) are sufficiently similar to intertidal microgeographic studies to warrant the suggestion that the same compensatory phenomenon is involved. High intertidal individuals, similar to warm seas populations, behave as though they are warm-adapted relative to low intertidal individuals and cold seas populations. Rao (1953), and Segal, Rao and James (1953), in the only studies where microgeographic and macrogeographic physiological differences have been compared in the same species (*Mytilus californianus*), show that 2½ feet of vertical separation is equivalent to about 350 miles in latitudinal separation. The rate of water propulsion in low-level northern mussels differs as much from that in low-level southern mussels as the rate in low-level southern mussels differs from that in high-level southern mussels 2½ feet higher in the intertidal zone. The data suggest that we are dealing with the phenomenon of individual adjustment to habitat temperatures; in short we may hypothesize that this is a phenotypic adaptation. In the present study we have made a more direct test of this hypothesis.

If a physiological rate character is, in time, readily reversible under changed temperature conditions, we may say that this rate attribute is acquired during the ontogeny of the individual. These changed temperature conditions may be artificially imposed by laboratory acclimation or by transplantation of the organism in the field; they may be naturally imposed by the changing season.

When low-level *A. limatula* were transposed to high-level, slowing of the heart rate was evident in two days and full adaptation was accomplished within 14 to 29 days. The reverse, adaptation to cold, was also complete within 14 to 29 days (Figs. 3, 4, and 5). Thus, the difference in heart rate of individuals at different intertidal levels was shown to be reversible under habitat conditions.

There are few published reports on transplanting individuals of a species from one habitat to another, using some physiological rate character as a measure of adjustment. Sumner and Lanham (1942) and Loosanoff and Nomejko (1951) report instances of transplantation with no apparent acclimation. These results may be due either to the inability of a homogeneous species to acclimate as in the first reference cited, or to the existence of true physiological races as in the second reference. Physiological races have been demonstrated previously both among field and laboratory populations (Brown, 1929; Goldschmidt, 1932, 1934; Hovanitz, 1947; Stauber, 1950). The transplantation method seems to be effective for revealing the nature of intraspecific physiological and morphological differentiation (Moore, 1934; Segal, 1956).

Acclimation of the heart rate has also been shown to occur with the seasonal change in temperature. Low-level animals have about the same rate in winter and in summer at their respective field temperatures (Fig. 7). High-level animals

show responses to unseasonal air temperature fluctuations which tend to mask the seasonal acclimation (Fig. 8). In substantiation of the field studies, acclimation to cold has also been demonstrated in the laboratory (Fig. 6).

Temperature sensitivity. The sensitivity of the heart rate to temperature change, measured by Q_{10}, has also been shown to vary with intertidal height. Between 9° and 19° C., but not as clearly above 19° C., low-level, cold adapted organisms show lower Q_{10}'s than equal weight high-level organisms (the rate of change over temperature *intervals* is used rather than at temperature points because it is believed that rates of 1° C. increments are necessary for a reliable estimation of the change in rate at a given temperature). Bělehrádek (1935), using examples taken from data of various investigators, points out that temperature coefficients commonly increase with the adaptation of the protoplasm to higher temperatures. This thesis is further strengthened through additional evidence of Rao (1953) and recalculations by Rao and Bullock (1954) of earlier equivocal data.

Of interest are the temperature relations of high and low intertidal groups. Although low-level animals respond as though they are cold acclimated relative to high-level animals, environmental temperatures below 13° C. are probably rarely encountered in this area. High-level animals do meet with such temperatures during the winter and spring months when the higher of the two low tides and the lower of the two high tides of each day are of insufficient magnitude to cover the animals. It is worthy of note that on the two occasions when heart rates were measured at 7° C. (at 4° C. both groups show cold depression and cessation of beat in a fair percentage of each sample), low-level animals show higher Q_{10}'s between 7° and 9° C. It indicates that the low-level, cold acclimated group is paradoxically approaching cold depression at a higher temperature than the relatively warm acclimated, high-level group. It further suggests that the physiological temperature range (that range of temperatures over which there is no observable indication of depression) extends farther into the cold in the warm acclimated group. Above 29° C. heart beats could no longer be counted with accuracy, but the very fact that high-level animals have been found with higher body temperatures in the field, while the surface ocean temperature rarely if ever has exceeded 24° C. in this locale, permits the interpretation that the physiological range similarly extends farther into the warm. Dehnel (1955) reaches the same conclusion for optimal temperature range of larval growth within the species in populations from Southern California and Alaskan waters.

Additional confirmation exists for the thesis that cold-adapted organisms show lower Q_{10}'s and thus greater independence to temperature change. Winter animals as compared with summer animals have lower Q_{10}'s, at least from 9° to 24° C., and animals transplanted from low-level (cold) to high-level (warm) show an increased temperature dependence (higher Q_{10}'s) within two days.

Scholander *et al.* (1953), in a metabolic study of arctic and tropic poikilotherms, suggests that a low Q_{10} would only be advantageous to offset the effects of changes in temperature due to diurnal, seasonal, or migratory factors. The authors state that no such adaptation was found among the species which would profit from a low Q_{10}: temperate water forms, fresh water forms, and terrestrial forms. Rao and Bullock (1954) agree that at present no general case can be made for lower Q_{10}'s in forms exposed to changing temperature but argue that cold adapted, *e.g.*, arctic

species as compared with tropic species, do show lower Q_{10}'s even though they may not be normally exposed to changes in temperature.

The present study is unique in that comparisons are made between animals which are living under fluctuating temperature conditions (high-level, exposed) and animals which are living under relatively constant temperature conditions (low-level, submerged). In this locale the high-level forms are exposed to considerably higher temperatures than that of the ocean and these animals act as though warm adapted. As shown, the warm adapted animals have the higher Q_{10} in spite of the fluctuating temperature of the habitat. Similarly, the summer forms from both high- and low-level have higher Q_{10}'s.

These differences could not have been expected simply from measurements of the Q_{10} of the *species* at any one time. If individuals of a species residing in different microhabitats and from one season to another show variations in temperature sensitivity, a great burden is placed upon comparisons between species. Other than for species living in arctic and tropical seas, with their almost constant temperatures, it is doubtful whether Q_{10} values (or any temperature coefficient describing sensitivity to changes in temperature) of a physiological rate activity are meaningful except in very limited comparisons. The thermal history of one segment of a species is not the thermal history of that species. The range of Q_{10} values (at a given temperature) permissible within the genetic makeup of a species would describe the temperature sensitivity of that species.

Spawning and gonad size. If the data on spawning represent normal behavior, then low-level *A. limatula* spawn before high-level *A. limatula*. Now the question remains as to whether high-level *A. limatula* actually spawn. All low-level animals show partial or complete spawning by late August. High-level animals, on the other hand, if they do spawn, do so sometime between October and December. We are not sure that high-level animals spawn because gonadal turgor and deformation of the female gametes, which Fritchman (1953) considers as indicative of pre-spawning ripeness, were not present in October although the gonads of high-level animals were of large size. Therefore, we must assume either that (1) high-level animals do not spawn in the field, or (2) pre-spawning ripeness is not a necessary condition. Again, if spawning occurs, it is out of phase with that of the low-level population. Yet, Fritchman (1953), working with high- and low-level members of two species of the same genus in central California (*A. fenestrata cribraria and A. testudinalis scutum*), did not find a difference in spawning time.

With the warming of the ocean in May and June (Fig. 8), gametogenesis is stimulated in both high- and low-level populations. By July, all animals showed the gonadal turgor and deformation of the female gametes associated with the pre-spawning ripe condition. Spawning occurs in the low-level population; this population is submerged and therefore subjected to the more constant temperatures of the ocean. High-level animals, which are only submerged 50% of the time, do not spawn during this period of warmest average sea water temperatures (July, August, September: 21°–19° C.). But, by October, the characteristics associated with pre-spawning ripeness have disappeared although the gonads are still large. From October to December, when the average inshore surface water temperatures have fallen to 17° C. and below, the high-level animals lose their gonads. The trigger mechanism necessary to initiate spawning may well be a required time interval spent

at a given temperature rather than the reaching or exceeding of that temperature for a short interval of time. Fifty per cent of the high-level animals (September collection; mean ocean temperature 19° C.) spawned in the laboratory after three days at 17° C.

The loss of the gonad sometime between October and December coincides with the seasonal tidal change; from October through April high-level animals are exposed during the late morning and afternoon hours (Fig. 8). It is during these hours that these animals are subjected to direct solar radiation and to heat conduction from the exposed rock substratum. The presence of a large gonad, or for that matter any gonadal material, would decrease the area under the shell available for water and thereby decrease the animals' ability to avoid desiccation (Segal, 1956). Low-level animals transplanted to high-level lost the gonadal material within two weeks (Table V). The evidence suggests that we are dealing with a non-breeding population living at the extreme of the intertidal distribution of the species.

Summary

1. Highest and lowest members of a eurytopic intertidal species, *A. limatula*, have been compared in the following: heart rate, gonad size, and spawning behavior.

2. Within the weight range of 0.4 to 1.0 gm., the heart rate varies inversely with increasing weight. The regression coefficients fall between -0.042 and -0.172; thus no single expression is available to describe fully the effect of weight on heart rate in this species.

3. Sex and size of gonad (as divorced from turgidity) have not been found to contribute to the variation in heart rate between samples.

4. Comparing equal weight animals, it is found that low intertidal individuals have faster heart rates than high intertidal individuals at any temperature from 4° to 29° C.

5. From data on field temperatures it is suggested that the significant parameter of the intertidal difference is temperature. High-level animals are subjected to considerable periods of warmer as well as to some periods of cooler temperatures than are low-level animals.

6. An attempt was made to characterize the difference in heart rate by: transplanting the animals in the field, following the seasonal changes, and maintaining samples of both populations in the laboratory at a cool temperature (14° C.) and without food.

7. When low-level animals are transplanted to high-level their heart rates slow so that within 29 days it is equal to that of the high-level animals when measured at any given temperature. The half-acclimation time was about 6 days. In the field, acclimation to cold was also shown to be complete within 29 days.

8. Comparisons of the heart rate during winter and summer showed that both high and low intertidal animals have faster rates in winter at any given temperature from 9° to 29° C. Acclimation to cold was also shown in the laboratory.

9. The above results lead to the interpretation that the microgeographic difference in heart rate is a phenotypic expression of a compensatory phenomenon operating to maintain approximately equal heart activity in spite of the habitat temperature differences. Latitudinal differences in physiological rate activities which

are clearly correlated with habitat temperature are sufficiently similar to the intertidal differences reported here to warrant the suggestion that the same phenomenon is involved.

10. Low-level and winter animals show a heart rate that is less dependent on temperature changes in the range from 9° to 19° C. This same response is not observed consistently above 19° C. While both high- and low-level animals appear to be approaching cold depression below 9° C., the low-level animals are cold depressed at a higher temperature than the relatively warm-adapted high-level animals; the low-level animals possess a higher Q_{10} in this range. It is suggested that the physiological temperature range of the warm-acclimated group extends both higher and lower than that of the cold-acclimated group. In the field, the Q_{10} of the heart rate changes within two days after the animals are transplanted.

11. The size of the gonad also varies with intertidal height. Low-level animals maintain a larger gonad during winter and spring than do high-level animals. Transplantation also reveals this difference to be reversible.

12. Analysis of spawning behavior (using turgidity as the criterion of pre-spawning readiness) presents the possibility that either (1) the two groups spawn a number of months out of phase with each other, or (2) high-level individuals do not contribute to the breeding population.

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MEMBRANE POTENTIAL AND RESISTANCE OF THE STARFISH EGG BEFORE AND AFTER FERTILIZATION [1]

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Unequal distribution of ions between the interior and exterior is characteristic of living cells. In many thus far studied the concentration of potassium is much higher in the interior while the concentrations of sodium and chloride are lower. This ionic asymmetry is associated with a potential difference across the plasma membrane which is approximately related to the relative concentration of potassium (Höber, 1945; Hodgkin, 1951) according to the Nernst equation:

$$E = \frac{RT}{nF} \ln \frac{(K^+)i}{(K^+)_0} \qquad \text{or at } 20^\circ \text{C.,} \qquad E \text{ (in mv.)} = -58 \log \frac{(K^+)i}{(K^+)_0}.$$

Membrane potentials have been recorded from many cells (Hodgkin, 1951; Grundfest, 1955), including some whose internal potassium concentration is known, by means of a fine, saline-filled, microcapillary (Gelfan, 1927, 1931; Ling and Gerard, 1949) inserted through the cell surface. The magnitude of this potential in different cells ranges from 50 to 100 mv., inside negative. This indicates an internal excess of potassium approximately 9 to 50 times the external concentration, and is in approximate accord with the observed values in specific cases in which potassium concentration has been determined.

Several investigators (Gelfan, 1931; Rothschild, 1938; Kamada and Kinosita, 1940) had, many years ago, reported that they could find no potential difference across the membrane of echinoderm eggs.[6] Interest in this problem has sharpened recently because of two new factors. In the first place a number of workers (Scheer,

[1] This work was reported at the General Scientific Session of the Marine Biological Laboratory in 1955 (Grundfest, Kao, Monroy and Tyler, 1955; and Tyler, Monroy, Kao and Grundfest, 1955). We wish to thank the Director and Staff of the MBL for the facilities placed at our disposal.

[2] Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena. Work of this investigator supported in part by research grant C-2302 from the National Cancer Institute, National Institutes of Health, Bethesda, Md.

[3] Istituto di Anatomia Comparata, University of Palermo, Italy. Fullbright Fellow, Summer, 1955.

[4] Department of Physiology and Pharmacology, State University of New York, College of Medicine at New York.

[5] Department of Neurology, College of Physicians and Surgeons, Columbia University, New York. Supported in part by grant (No. 1880-Penrose) from the American Philosophical Society; and grants from Muscular Dystrophy Association of New York, and Process and Instruments, Brooklyn, N. Y. Laboratory facilities were provided by a grant from the MBL under its ONR contract (Monr—09703).

[6] In a brief report Taylor and Whitaker (1926) mention experiments on eggs of the sea urchin *Clypeaster rosaceus* showing a potential difference, inside negative, of about 1 mv., which would be very low in comparison with other kinds of cells that have been investigated.

Monroy, Santangelo and Riccobono, 1954; Furshpan, 1955; Kao, 1955) have independently made similar observations in several varieties of marine eggs, using modern recording equipment and stable KCl-filled (Kamada and Kinosita, 1940; Nastuk and Hodgkin, 1950) microcapillary electrodes. In the second place, convincing data have become available (Rothschild and Barnes, 1953) showing that at least for the eggs of the sea urchin *Paracentrotus lividus*, the potassium content in the aqueous phase of the egg is 21 times higher than in sea water, while the internal sodium and chloride concentrations are, respectively, about $\frac{1}{9}$ and $\frac{1}{7}$ of those in sea water. It would therefore seem likely that in the sea urchin egg a membrane potential of about 80 mv., inside negative, should be observed.

The persistent failure to find a membrane potential prompted a re-examination of this problem with certain technical refinements which provide definitive verification of the entry of a microelectrode into the cell, as well as measurements of the resistance and capacity of the membrane. Parallel experiments with microinjection (Tyler and Monroy, 1955) helped to elucidate and overcome difficulties encountered in attempts to pierce the cell membrane of echinoderm eggs. A potential difference was thereupon found to exist across the membrane of *Asterias* eggs. Its magnitude was found to be somewhat lower than would be expected on the basis of the high internal K^+, which was also determined in these experiments. As in other kinds of cells that have been investigated, the membrane potential difference changes reversibly on changing the external K^+ concentration.

Although, as will be shown below, it is unlikely that penetration by the microelectrode had been attained in earlier work, several observers (Rothschild, 1938; Scheer *et al.*, 1954; Furshpan, 1955) have reported that eggs could be fertilized while apparently impaled. Fertilization was also successful in the present experiments with the electrode truly inside the egg. The effects of fertilization on the potential and on the electrical constants of the membrane were therefore also studied.

Methods

Tyler and Monroy (1955) carried out experiments attempting microinjection of fluids into eggs of *Arbacia*, *Echinarachnius* and *Asterias*. A smaller number of experiments were performed in the present series in an effort to penetrate eggs of *Arbacia punctulata* with microelectrodes. Confirming the experience of Chambers, (Pandit and Chambers, 1932, and personal communication) in both cases it was found that piercing the surface is difficult. Microelectrodes or micropipettes which appear to have penetrated, in actuality only carry the membrane before them even to the extent of creating a tunnel (as Dan, 1943, has also observed) as the microcapillary travels through the diameter of the egg. This was clearly revealed in the microinjection experiments of Tyler and Monroy (1955), in which it was observed that the membrane could form a tight sleeve around the inserted pipette, the latter then appearing to be within the cytoplasm of the egg. However, injected fluid (KCl-NaCl solutions) would simply expand this sleeve and flow out into the surrounding medium rather than into the egg. Eggs of *Asterias forbesii* behaved similarly, as illustrated in Figure 1, but in view of their larger size (average diameter, 146 μ) these were chosen in preference to eggs of *Arbacia* for further investigation. Penetration of these eggs was accomplished by the technique of jarring the preparation by a light tap on the table. This sudden vibration was especially ef-

fective after the indented plasma membrane had formed a tight sleeve around the electrode (as in Figure 1e) and was allowed to remain in this condition for a short while. Figure 2 illustrates eggs with one or two electrodes that have penetrated into the cytoplasm.

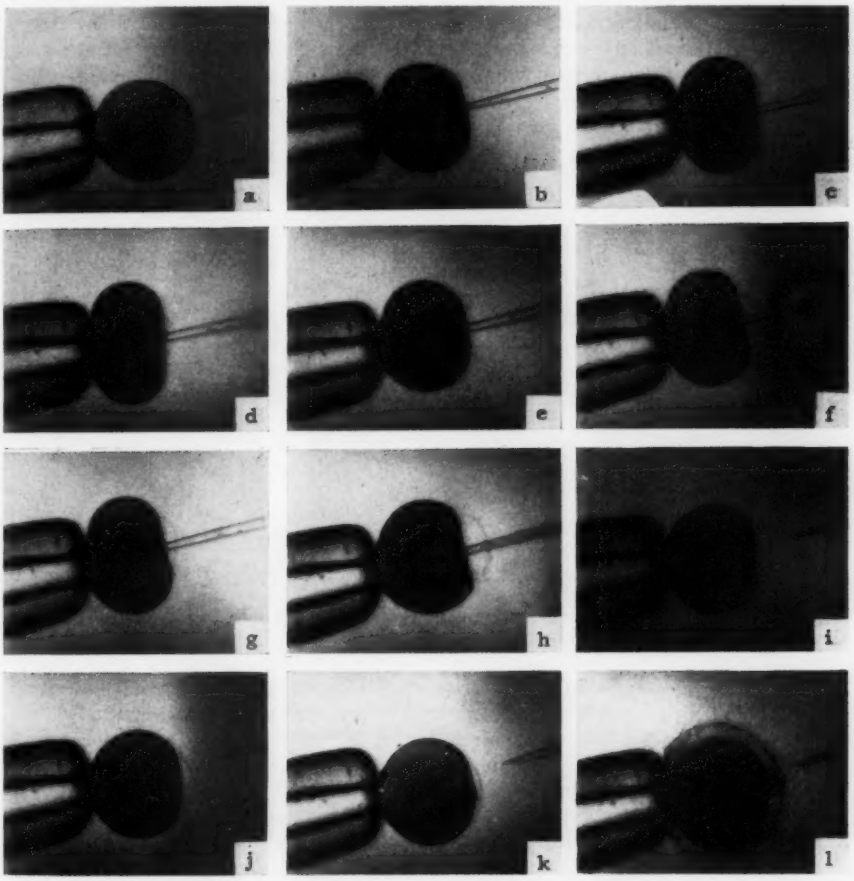

FIGURE 1. Photomicrographs showing apparent entry of a micropipette into an egg of *Asterias forbesii* held by a "sucking" pipette. Magnification, 104×. The micropipette, filled with isotonic NaCl–KCl solution containing chlorphenol red, is pushed through the vitelline membrane and indents the underlying surface (plasma membrane) forming a large conical depression (*d*). After about two minutes the depression closes over the pipette (*e*) and the latter appears to be within the cytoplasm. However, injection of fluid (*f* to *h*) shows that the walls of the depression had formed a tight sleeve around the shaft of the pipette. The fluid expands this sleeve and stretches the vitelline membrane, flowing out through the latter. Upon removal of the pipette (*i* to *k*) the remaining fluid is expelled as the egg rounds-up within some two minutes. The same egg with a fertilization membrane elevated at two minutes after fertilization is shown in *l*.

The experiments were carried out primarily on unfertilized eggs. A few measurements were made on eggs with germinal vesicles or fertilized prior to impalement by microelectrodes. The eggs were obtained from spontaneously shedding animals. They were kept at temperatures of 18 to 20° C. until used, one to five hours after shedding. The experiments were carried out mostly at about 25° C. and all the eggs used appeared to be normal. All those so tested, as well as parallel samples, were fertilizable. Some batches of eggs were stored at about 10° C. but these underwent spontaneous activation on transfer to the room temperature and were, therefore, not used in the experiments.

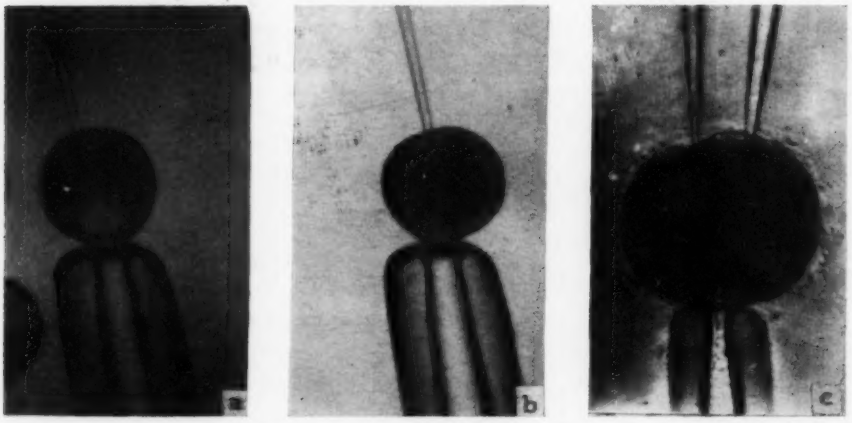

FIGURE 2. Photomicrographs of eggs of *Asterias forbesii* held on "sucker" and impaled on microelectrodes. Magnification: *a* and *b*, 116×; *c*, 200×. *a*, an egg with intact germinal vesicle; *b*, another after dissolution of the germinal vesicle; *c*, an egg fertilized after insertion of two microelectrodes. The tips of the microelectrodes are not visible.

All the impaled eggs, in the experiments involving fertilization, had undergone dissolution of the germinal vesicle, and were in various stages of the maturation divisions. Sperm were diluted in a solution of 10^{-3} molar Versene [7] in sea water, since the latter improves the fertilizing power of dilute sperm suspensions (Tyler, 1953). The sperm were introduced by means of a capillary pipette at a distance several millimeters from the egg. The time at which sperm were seen to approach the egg, as well as the time of formation of the fertilization membrane were noted for correlation with the measurements of the membrane potential.

Experimental arrangement

Sea water containing eggs was placed (Fig. 3A) on a transilluminated lucite plate mounted on a mechanical stage under a binocular microscope. The sea water was in continuity with one end of a sea water-filled tunnel, into the other end of which was inserted an Ag-AgCl reference electrode. One or two microelectrodes (tip diameters less than 0.5 μ), each individually carried in a micromanipulator, ap-

[7] Versene is the trade name (Bersworth Chemical Co.) of ethylene diamine tetraacetic acid.

proached diagonally from above at a small angle. Opposite was another manipulator which held a glass suction pipette. This device (Tyler, 1955b), modified from the elastimeter of Mitchison and Swann (1954), was very useful for holding the egg fixed gently but firmly at one pole while the microelectrodes were pressed against the other (Figs. 1 and 2). The polished tip of the "sucker," somewhat smaller than the diameter of the egg, dipped into the sea water containing the eggs. The other end was flexibly coupled to a vertical glass tube which could be raised or lowered by a rack and pinion movement. The system was filled with sea water. By maneu-

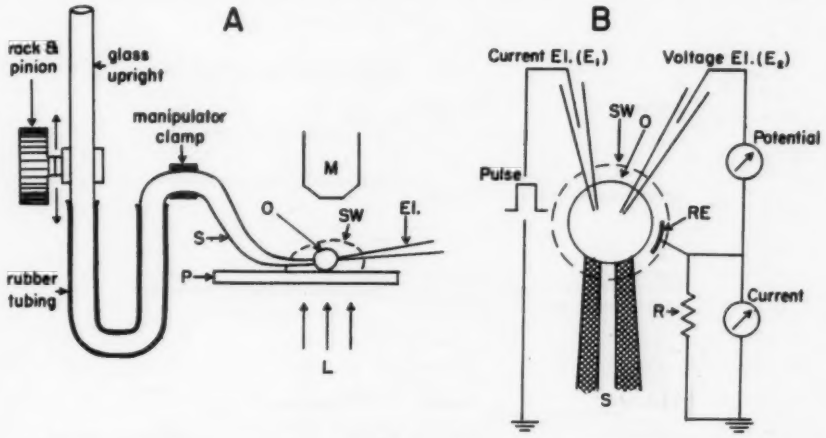

FIGURE 3. Diagrammatic illustration of the experimental arrangement. *A:* The mechanical and optical set-up. The egg (O) is shown (enlarged) lying in a drop of sea water (SW) on a lucite plate (P) which is illuminated from below (L) and observed through a microscope (M). The sucker (S) has a rack and pinion for raising or lowering the vertical tube. The electrodes (E) are inserted into the egg at the pole opposite that held in the sucker. *B:* The electrical arrangement. The two microelectrodes ($El._1$ and $El._2$) are shown in the egg (O). $El._1$ is the current electrode fed through a pulse generator. $El._2$ is connected to one grid of the potential recording amplifier. The other grid connects with the fluid (SW) as well as with a resistor (R) through the reference electrode (RE). Across R is the amplifier measuring the current in the pulse. When a single microelectrode ($El._2$) was used, RE was grounded.

vering the mechanical stage, any desired egg in the drop could be brought to the vicinity of the tip of the sucker. Lowering the upright created sufficient negative pressure to take up and hold the egg firmly against the tip. Manipulation was carried out under 80 or 160 × magnification.

Electrical measurements

Determination of the membrane potential and the resistance and capacity of the membrane constituted the electrical measurements. For the former a single microelectrode, drawn prefilled with 3 *M* KCl (Kao, 1954), was sufficient. This was connected to a high impedance negative capacity input amplifier [8] and a cathode ray

[8] Designed by Mr. E. Amatniek, electronic engineer at the Dept. of Neurology, Columbia University.

oscillograph. The external medium was grounded through the Ag-AgCl reference electrode, or in some cases the "sucker" was itself made the reference system. The standard sensitivity employed was 20 mv./cm. deflection on the face of the oscillograph tube so that changes in potential as low as two to three mv. could be detected. Visual observation was supplemented by photography of the trace.

Determination of the electrical constants of the membrane required passage of a square pulse current through the membrane and the measurement of the potential difference created by this current across the resistance and capacity of the membrane (Fig. 3B). For this purpose two microelectrodes were inserted into the eggs (Fig. 2c). One of these was connected to a pulse generator delivering 12 or

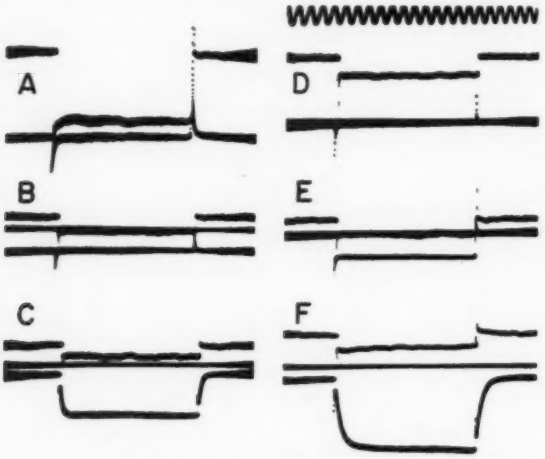

FIGURE 4. Current and voltage pulses as recorded before and after entry of two microelectrodes into the egg. *Left:* A large current pulse (*A*, upper trace) caused only transient capacitative artifacts in the voltage trace (below) as long as the electrodes were outside the egg. *B, C:* The current pulse was reduced. *B*, before and *C*, after penetration of the electrodes. A third trace, which represents the zero level at the time of entry, is seen at its correct position in *C*. The membrane potential, about −20 mv., has brought the voltage trace down. The square pulse of current is reflected in a membrane *IR* drop with retarded onset and decay. *Right:* Another egg. *D:* The current pulse caused only the capacitative artifact on the voltage trace. *E:* When the two electrodes were pressed against the egg, the voltage trace also recorded a deflection with rapid onset and decay. However, the steady potential was zero. *F:* A few seconds later, the electrodes had penetrated the egg, causing the characteristically slowed onset and decay of the voltage trace. A membrane potential of about −30 mv. is also seen. Time scale in msec., upper right.

30 msec. pulses of controllable amplitude, synchronized in rate and time with the sweep of the oscillograph. The external reference electrode was connected to a resistance, the other end of which was grounded, as was the return of the stimulator. An amplifier across the resistor recorded the *IR* drop in the latter and the current, *I*, through the membrane was calculated from this measurement. The sensitivity of the current trace on the oscillograph was 1 mv./cm. and with a 1 megohm resistor for the *IR* drop this amplitude of deflection corresponded to 0.001 μA.

The second microelectrode led, as before, to another amplifier, but in this case recording the membrane potential differentially, the second grid being connected to the indifferent electrode and the high end of the resistor. The potential change of this amplifier during the square pulse thus represented the IR drop across the membrane in series with the resistance of the fluid. Since the resistance of the latter was small compared with that of the membrane, it was neglected. From the knowledge of I obtained in the current record, R of the membrane could be computed. This was transformed to the specific membrane resistance R_M (ohm-cm.^{2}) by multiplying by the surface area of the egg (average diameter = 146 μ; surface = 6.7 $\times$

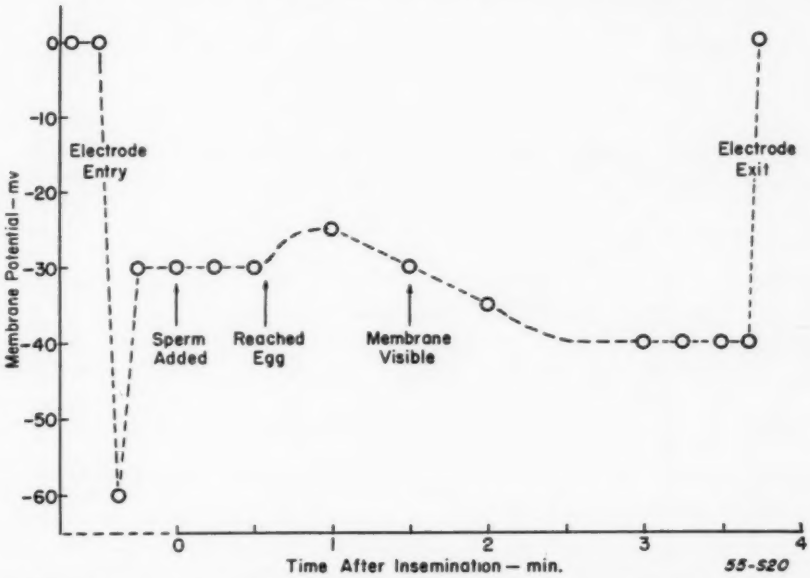

FIGURE 5. Membrane potential of Asterias egg and its changes on fertilization. Entry of the electrode into the egg caused sudden appearance of -60 mv. membrane potential, which decreased to -30 mv. rapidly. The time scale has its origin at the time sperm were added to the sea water. Within 30″ sperm were seen to have contacted the egg and at this time the previously steady potential decreased by 5 mv. Subsequently the potential again increased and remained steady at -40 mv. until the electrode was removed. Absence of drift in the system is indicated by the return of the voltage trace to the initial value.

10^{-4} cm.^{2}). The time constant (τ) when the rise and fall of the voltage trace (Fig. 4C, F) had reached 67% of the final value was measured from the records. The membrane capacity (C_M) was determined in μF/cm.^{2} from the relation $\tau = R_M C_M$.

Certain precautions have to be taken in experiments of this type. The current electrode must be non-polarizable in the range of currents used for the measurements. This was checked at the start and end of each experiment. Secondly, applied current must be rather low. With a microelectrode tip of 0.5 μ diameter, 1 μA flow represents a current density at the tip of about 500 A/cm.^{2} which might lead to

heating and perhaps breaking of the electrode. Furthermore, a flow of 1 μA through the whole surface area of the egg membrane is equivalent to a current of 1.5 mA/cm.^{2} which is a high density, at least for the membranes of excitable tissues.

The use of the two microelectrodes and a current pulse served the additional important purpose of providing unequivocal evidence of the penetration of both electrodes into the egg (Fig. 4). When these electrodes were in the fluid the record of the voltage trace differed radically from the trace of the current pulse (Fig. 4A, B, D). The former showed only rapid short-lived deflections of opposite sign at the beginning and end of the applied pulse. These are attributable to capacitative

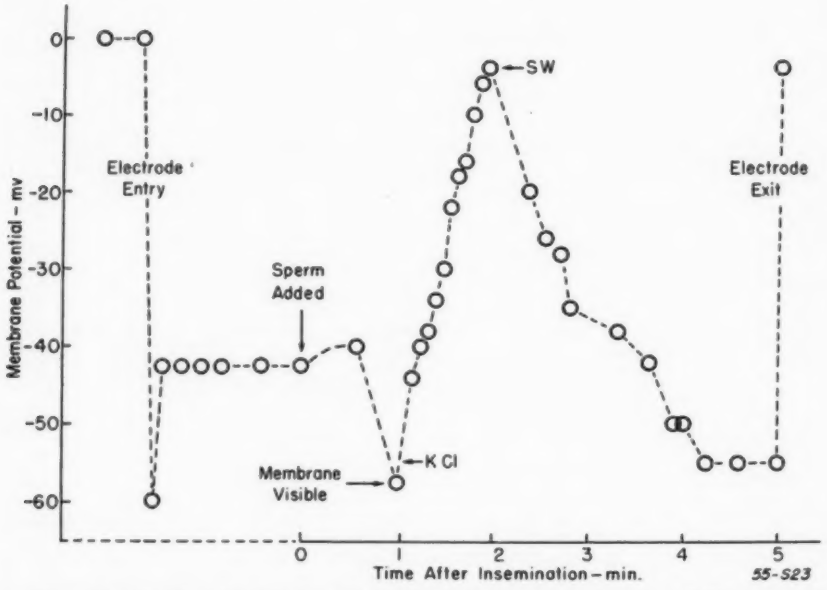

FIGURE 6. Depolarizing effect of externally applied KCl and its reversibility. The sequence up to one minute on the time scale is similar to that of Figure 5. When the membrane potential of the fertilized egg had reached its maximum value, the sea water was largely replaced with isotonic KCl. This caused rapid and almost complete depolarization, which was reversed on washing out the KCl with sea water. A drift in the amplifier of 4 mv. negative had changed the base line slightly.

coupling between the electrodes. During the major portion of the applied pulse, the voltage trace remained essentially at zero potential, reflecting the low resistance of the sea water and the consequently low IR drop across it. On occasion, when both electrodes were simultaneously pressed firmly against the egg, the voltage trace showed a large deflection which would be expected if the current path now included a high resistance formed by the surface of the egg membrane. However, this voltage record was characterized (Fig. 4E) by its faithful reproduction of the form of the current pulse, indicating that the recording electrode was not across

the capacity of the membrane. When penetration occurred the voltage pulse changed in form characteristically, rising and falling more slowly than the current trace (Fig. 4C, F). This behavior, in view of our original uncertainty as to the existence of a potential difference across the egg membrane, proved valuable initially in definitely establishing the entry of the microelectrodes into the cytoplasm.

Ionic content of Asterias eggs

Measurements of the internal ionic milieu of *Asterias* eggs were not found in the literature. A sample of over two million eggs was therefore subjected to analysis by flame photometry. The procedure is detailed below in conjunction with the data obtained.

Results

Membrane potential

In nearly all the eggs studied no potential accompanied apparent penetration by the electrode, but one or more taps on the table always caused the sudden appearance of a potential difference with the internal electrode negative, except in a number of eggs which cytolyzed as the electrode appeared to enter. The failure to obtain the

Table I

Steady potential difference observed across the membrane of unfertilized eggs

P.D. in mv., inside negative	10	15	20	25	30	40	45	50
Number of eggs (Total 24)	3	2	5	1	8	3	1	1

In one additional egg, fertilized prior to penetration of the electrode, the membrane p.d. was initially −40 mv., and increased to −48 mv.

In one other egg only a p.d. of +10 mv. was obtained.

potential initially was probably due to extensibility of the egg membrane. Jarring probably caused penetration of the membrane during the resultant vibration. The potential, upon penetration, reached values up to 60 mv., apparently instantaneously, then rapidly declined to a lower steady value (Figs. 5, 6). The steady value of the membrane potential ranged in different experiments from a low of 10 mv. to a high of 50 mv. (Table I), with the majority of eggs showing potentials of 20 to 30 mv.

The larger initial value probably reflects more nearly the true potential difference momentarily disclosed as the fine tip of the microelectrode broke through the egg membrane. The subsequent release of tension of the latter, as it rounds up and moves farther onto the electrode, could create an imperfect seal of the membrane around the shaftlet and lead to partial short circuiting of the full potential. In most experiments the potential remained steady at the lower value as long as the electrode was left in the egg. Withdrawal was accompanied by an abrupt return of the oscillograph trace to the zero potential level. There seemed to be no consistent difference in the value of the steady potential if two electrodes were inserted simultaneously or sequentially. The number of experiments of this type was too

small and the scatter of potentials too great to employ this method for calculating the possible magnitudes of leaks around the electrode.

Two eggs included in Table I, through which relatively high currents (0.5 to 1.0 μA) were later passed, cytolyzed in the course of the experiments. Five cytolyzed spontaneously, and two that had an intact germinal vesicle cytolyzed on subsequent penetration of this structure. In all cases cytolysis resulted in disappearance of the membrane potential. In three cases the potential disappeared with no observable cytolytic effects. Movement of the tip then again disclosed the membrane potential. These can be interpreted as eggs in which the cytoplasm had become sealed off from the electrode (Chambers, 1922) by a precipitation membrane (Heilbrunn, 1927, 1952; *cf.* Costello, 1932). In a few other experiments, all done with the same microelectrode, penetration was indicated at first by a small positive potential (about 10 mv.) which in all but one case reversed to negativity. The positive potential may have been due to increase of the electrode resistance by plugging of the tip as this pressed into the egg membrane. With the grid current of the amplifier about 10^{-11}A and positive, a shift of + 10 mv. would be caused by insertion of a resistance of 10^9 ohms. Another possibility is that the electrode had penetrated the egg, but had been sealed off from the cytoplasm while some leakage remained around the shaft. The internal negativity would then register as positivity on pickup by the external electrode.

Membrane potential upon fertilization

Eight eggs were fertilized while impaled. Insemination was done usually at least 5 minutes after impalement in order to ascertain that the membrane potential was steady and that the egg was not undergoing cytolytic changes. When spermatozoa were seen to have reached the impaled egg (about 15 to 30 seconds after insemination) the membrane potential suddenly decreased from its previously steady value. This change amounted to 5 to 10 mv., and was temporary (Figs. 5, 6). The membrane potential then began to increase gradually, eventually attaining a magnitude greater than the former steady value, and in some cases as large as that momentarily seen during entry of the electrode. This increased internal negativity persisted during the subsequent period of observation which, for the present series of experiments, was not longer than 5 minutes. The new steady value of potential, 5 to 20 mv. higher than before fertilization, was attained in 1 to 2.5 minutes, which was also the time at which the fertilization membrane had become distinctly elevated.

Ionic content of Asterias eggs

Estimate of the magnitude of the membrane potential to be expected requires knowledge of the ionic concentration in the egg. Determinations were therefore made of the K and Na content of *Asterias* eggs. The procedure was as follows:

A 100-ml. suspension of unfertilized eggs was prepared in sea water. From this, a one-ml. sample was removed by means of a wide-mouth (2.5 mm.) pipette. It was diluted 20-fold and a one-ml. portion used for counting the number of eggs. During these procedures precautions were taken to keep the suspension of eggs distributed as uniformly as possible. The final one-ml. diluted sample contained

1096 eggs. Therefore the 99 ml. of the original suspension contained 2.17×10^6 eggs. The eggs of the latter suspension were allowed to settle, and most of the fluid was drawn off. The remainder was then centrifuged for 15 minutes at $1500 \times g$, in graduated centrifuge tubes. The packed eggs measured 6.1 ml., and above them was an additional gelatinous, opalescent layer of 2.1 ml. representing the material of the gelatinous coat of the egg. Supernatant fluid was withdrawn to leave a total volume of 10 ml. of packed eggs, gelatinous layer and sea water. The eggs lost during the procedure were determined from counts of aliquots of the

TABLE II

Measurements of diameters of eggs of Asterias forbesii

Number of eggs	1	3	1	1	2	2	1	3	Total: 14
Average of the two diameters (μ)	140	142.5	143.8	145	146.3	147.5	148.8	150	Average: 145.9

supernatants. Their number was 2128 or less than 0.1% of the total in the packed eggs. To the 10 ml. volume of packed eggs and supernatant, and separately to an equal volume of supernatant fluid, were added 10 ml. of sulphuric acid. The two preparations were allowed to stand overnight, transferred with distilled water washings to digestion flasks and boiled for about 4 hours, one ml. of 30% H_2O_2 being added to help clarify the material. Both preparations were then transferred to 100-ml. volumetric flasks and made up to that volume in distilled water. The original samples had thereby been diluted 10-fold. These were analyzed for K^+ and Na^+ by flame photometry.[9]

TABLE III

Determinations of K and Na content of eggs of Asterias forbesii

	Potassium	Sodium
(1) mM in 10 ml. of suspension containing 2.168×10^7 eggs	0.656	2.40 to 2.61
(2) mM in 10 ml. of supernatant	0.205	4.00 to 4.32
(3) mM in 6.47 ml. of supernatant	0.133	2.59 to 2.79
(4) mM in 3.53 ml. of eggs [(1)–(3)]	0.523	−0.19 to −0.18
(5) mM/ml. eggs	0.148	

To calculate the content of these ions in the eggs it was necessary to determine the egg volume, exclusive of interstitial fluid. The diameters of 14 eggs, in which the difference between diameters at right angles was less than 4%, were measured, with the results shown in Table II. The flame photometric determinations and calculated values of K and Na content are given in Table III.

On the basis of an average diameter of 146 μ the volume of each egg is 1.63×10^{-6} cm.^{3} and that of the total number in the suspension is 3.53 ml. The latter value is 58% of the volume (6.1 ml.) of the packed eggs after the low speed centrifugation, and is in reasonable agreement with the value to be expected from the packing of spheres, plus a small allowance for adherent jelly coat. The 10-ml.

[9] We are indebted to Dr. James Green of Rutgers University and to Dr. George Scott of Oberlin College for the analyses.

specimen containing eggs therefore was composed of 35.3% eggs and 64.7% interstitial fluid (including gelatinous coat material). From the measurements of the parallel sample of supernatant fluid the 6.47 ml. of the supernatant in the egg sample contained 0.133 mM K^+. The 3.53 ml. of eggs therefore contained 0.523 mM or 0.148 mM/ml. of eggs. This figure is about 15 times the K^+ concentration of sea water (0.01 mM/ml.). On the basis that the eggs contain approximately 75% water by weight and 80% by volume, the K^+ concentration becomes 0.185 mM/ml.

TABLE IV

Electrical constants of Asterias eggs

Expt. No.	Conditions	Steady membrane potential (mv.)	Maximum applied current (μA)		r_m (ohms)	R_M (ohm-cm.^{2})	τ (msec.)	C_M (μF/cm.^{2})
			Outward	Inward				
A. Maximum currents not exceeding .01 μA								
25	unfertilized	—*	.006	.006	4.0×10^6	2680	1.28	0.48
	fertilized	—	.006	.006	3.6	2410	1.25	0.52
26	unfertilized	−30	.006	.006	4.97	3330	1.92	0.58
27	unfertilized	−15	.005	.01	3.7	2430	1.04	0.43
28	unfertilized	−15	.005	.005	5.8	3880	2.75	0.71
	fertilized	−50	.005	.005	5.8	3880	1.6	0.41
B. Higher maximum currents								
7	unfertilized	−10	0.10	0.06	5.05×10^5	368		
8a	unfertilized	−10	0.10	—	11.	737		
b	unfertilized	−10	0.15	—	8.6	574		
11	unfertilized	−20	0.25	—	2.6	172		
6	unfertilized	−20	0.40	—	2.0	133		
13	unfertilized	−30	0.4	0.4	1.07	71.6		
1a	unfertilized	−30	1.0	1.0	2.6	174		
b	unfertilized		1.0	1.0	2.9	194		
14	unfertilized	—*	1.5	1.5	2.5×10^4	16.8		

* Potential not measured because of amplifier drift.

of the water in the eggs. This corresponds fairly closely with the value of 0.210 mM/mg. of water found in eggs of the sea urchin *Paracentrotus lividus* (Rothschild and Barnes, 1953).

The supernatant contained about the same amount of sodium as does sea water, but the potassium concentration (0.02 mM/ml.) was twice that of sea water (Table II). For the calculation given above it was assumed that the extra K^+ derived from gelatinous material of the eggs, some of which remained in the supernatant. On the other hand, if this potassium had leaked out of the eggs during preparation for analysis, the initial concentration of the ion in the eggs would have been 0.168 mM/ml. of eggs and 0.210 mM/ml. of the water in the eggs.

Similar calculations for the Na content of the starfish eggs, using either the values of 2.40 and 4.00 mM/10 ml. of egg suspension and supernatant, respectively,

or 2.61 and 4.32 gave a slightly negative value (− 0.2 mM per 3.53 ml. eggs) for this ion. In view of the high content of Na in sea water and therefore in the interstitial fluid of the egg suspension, the value for Na is much more sensitive to errors in determination of egg volume than is that of K. For example, if the actual egg-volume were 13% greater than determined, the calculated Na content would be zero while that of K would be 0.17 mM/ml. of the water in the eggs. We may therefore conclude that the Na concentration in *Asterias* eggs is less than one-twentieth, while the K concentration is between 17 and 21 times, the values found in sea water.

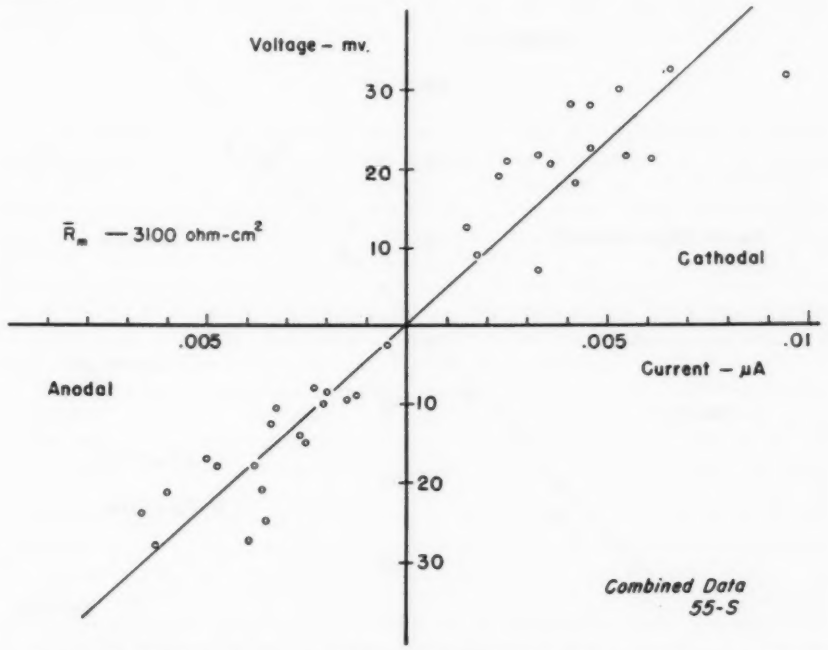

FIGURE 7. Membrane current-membrane voltage relation in four Asterias eggs. Two of the six experiments represent measurements done after fertilization. The slope of the straight line drawn through the combined data is the average resistance (r_m) and yields $R_M = 3100$ ohm-cm². *Anodal* signifies outward current and *cathodal*, inward.

Change in membrane potential on increasing external K^+

Four experiments served to test and demonstrate the sensitivity to K^+ of the potential difference across the egg membrane, but the relation between the external K^+ and the potential was not studied quantitatively. Within a few seconds after isosmotic KCl was added to the sea water surrounding an impaled egg the membrane potential decreased (Fig. 6) and reached almost complete depolarization. The K^+-rich solution was then replaced with sea water and the initial value of the membrane potential was again restored. Fertilized and unfertilized eggs responded

in the same manner. The technical arrangement did not permit rapid washing out of the excess KCl and this probably accounts for the slower return of the initial membrane potential.

Membrane resistance and capacity

These electrical constants of the membrane were determined in 10 eggs with the square pulse technique described earlier. However, in six the maximum current densities were rather high (0.5 to 1.5 μA) and the results were probably affected

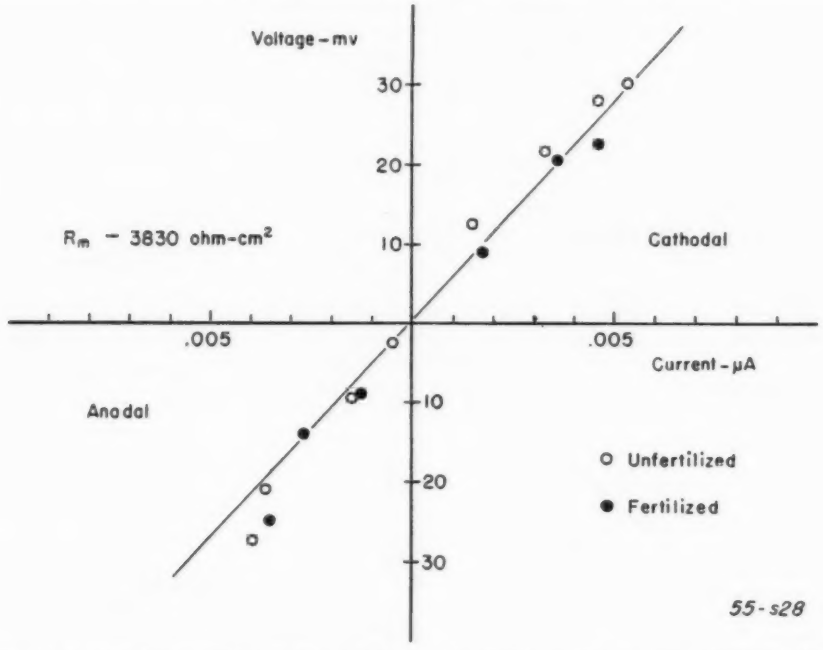

FIGURE 8. Membrane resistance before and after fertilization. The current-voltage relation of the same egg before and after fertilization.

by this condition. In six experiments with four eggs (two eggs were studied after fertilization as well as before) the maximum current of the square pulse was limited to 0.01 μA and these results are shown in Table IVA and Figures 7 and 8. The average membrane resistance was 3100 ohm-cm.[2] In the two eggs measured after the fertilization membrane was elevated there was no significant change in resistance (Fig. 8; Table IVA). In the six experiments employing high current densities (0.5 to 1.5 μA) the values of R_M were markedly smaller (Table IVB). No explanation will be attempted at present for this effect. Over the range of current densities used in all the experiments the relation between membrane voltage and current was linear. Assuming a specific resistance of 100 ohm-cm. (approximately

three times that of sea water; Cole and Cole, 1936a, 1936b) for the cytoplasm, the droplet constituting the interior of the egg would have a resistance of about 8×10^3 ohms, surrounded by a membrane with a resistance several orders higher than this in magnitude.

Determination of the time constant (τ) and of the membrane capacity (C_M) was only approximate, because the oscillographic records of the membrane voltage change were made on too slow a time base. The average capacity (0.52 μF/cm.^{2}) is somewhat smaller than, but of the same order of magnitude as, the 1.1 μF/cm.^{2} obtained by Cole and Cole (1936a) with eggs of *Asterias forbesii*, and 0.7 to 2.7 μF/cm.^{2} listed by Cole and Curtis (1950) for unfertilized eggs of other marine animals.

Discussion

The original object of this investigation was to seek an explanation for the reported absence of a membrane potential in some echinoderm eggs. On the basis of Rothschild and Barnes' (1953) finding that sea urchin eggs contain 21-fold higher concentration of K^+ than does sea water it was to be expected that a membrane potential of about 80 mv. ought to be present. A membrane potential has now been found in *Asterias* eggs. Its magnitude, at least 60 mv. under what we believe to be the optimum condition, is smaller by about 15 to 20 mv. than might be expected on the basis of the values (17 : 1 to 21 : 1) that we obtained for the ratio of K^+ in *Asterias* eggs to that in sea water. A similar discrepancy is usually found in nerve and muscle fibers (Hodgkin, 1951 : Grundfest, 1955).

The reversible depolarization of the egg membrane in response to increasing the external K^+ agrees with the behavior of other cells (Hodgkin, 1951), but the quantitative relation between potential and K^+ concentration was not tested in the present experiments. Another point of similarity relates to the low internal Na^+ concentration. *Asterias* eggs contain too little Na^+ for accurate measurement under the experimental conditions employed. Rothschild and Barnes (1953) found a concentration of 52 mM/kg. water in eggs of *Paracentrotus lividus* as compared with 485 for sea water, and the Na^+ concentration of various excitable cells is also considerably lower than in the fluid surrounding them (Hodgkins, 1951). It is thereby likely that an active transport mechanism exists in echinoderm eggs as it apparently does in other types of cells (*cf.* Brown and Danielli, 1954).

Rothschild (1938), and Kamada and Kinosita (1940) had considered, but rejected, the possibility that failure to obtain a membrane potential might be due to failure of electrode to penetrate the egg. Their decision was based on the apparent entrance of injection fluid into the egg. Furshpan (1955) believed that because in many experiments he had pushed the electrode clear through the egg, it must have been in the cytoplasm at some stage in the process and therefore considered his results to demonstrate absence of a membrane potential. However, as has been noted by Dan (1943) and by Tyler and Monroy (1955), the micropipette can readily tunnel through the egg, without entering the cytoplasm; the distended plasma membrane on one side simply joins that on the other and both are then perforated without injury to the egg which can later close the tunnel. It is pertinent to quote in this connection the remarks of Chambers (1922, p. 189) :

Pushing a pipette, especially a comparatively large one, into an egg cell frequently causes the surface of the cell to become invaginated and thus forms a deep pocket. The tip of the pipette, even if it should finally break through the surface, is apt to become separated from the protoplasm of the interior by the formation of a new surface film continuous with the original surface of the cell.

Chambers' conclusion was confirmed by Tyler and Monroy (1955, and illustrated in Fig. 1) and in the present experiments. It is now also well known, particularly from the work of Heilbrunn (1927, 1952; *cf.* Costello, 1932), that when the cytoplasm of eggs of marine animals, or of other cells, is brought in contact with Ca-containing solution a surface precipitation reaction occurs. The formation of such a precipitation membrane around the tip of the pipette might have been responsible for the lack of a membrane potential in the experiments of Rothschild (1938) in which injection tests indicated penetration. The absence of a potential in the experiments of Gelfan (1931), Kamada and Kinosita (1940) and Scheer *et al.* (1954) can also be attributed to failure of penetration or possible formation of a precipitation membrane.

A small positive potential was observed by Gelfan (1931) when the microelectrode presumably penetrated the germinal vesicle of the *Asterias* eggs. In our experiments, confirming Chambers (1921), puncture of the germinal vesicle invariably led to cytolysis of the egg. The sudden disappearance of the membrane potential when the germinal vesicle was impaled and cytolysis resulted, indeed served as additional verification of penetration in the experiments reported in the present paper. As noted, and discussed earlier, small positive potentials were occasionally observed in these experiments.

Although the absence of a membrane potential in echinoderm eggs reported by earlier observers is explained in the light of the present experiments, there remains the finding (Kao, 1955) that eggs of the killer minnow, *Fundulus*, do not exhibit a membrane potential. The precautions using two microelectrodes and an applied pulse, were also employed in those experiments to ascertain penetration of the egg membrane. However, the ionic composition of *Fundulus* eggs is unknown and the explanation for this different finding must remain in abeyance.

For the most part measurements of the membrane potential have been carried out on cells from tissue aggregates. Some data are, however, available for unicellular organisms. These are of interest not only because they provide a rather closer analog to eggs than do tissue components, but also because they, too, reflect the effects of improvements in technique. Telkes (1931) reported that amoebae have a membrane potential of 10 to 30 mv., inside negative. Buchthal and Péterfi (1937) found only small variable potentials of either sign (up to 3 mv.). Later, however, Wolfson (1943) succeeded in recording a membrane potential of up to 90 mv. in *Chaos chaos*. Dr. S. Crain, of the Department of Neurology, Columbia University (personal communication) has obtained similar values for the membrane potential in *Chaos chaos* and *Paramaecium*. As in eggs, it is difficult to penetrate the cell membrane and Wolfson used the device of applying negative pressure on the electrode, sucking the amoeba onto the shaftlet and eventually rupturing its cell membrane. Tauc (1953) found a membrane potential of 80 to 100 mv. in the plasmodium of a myxomycete. As in the *Asterias* egg, the high initial value decreased subsequent to penetration.

Effects of fertilization on the membrane potential

A second objective of these experiments was to examine whether or not changes in membrane potential accompany the events of fertilization. Péterfi and Rothschild (1935), using two small external electrodes placed on opposite sides of the surface of the frog egg, reported (p. 875) that "there are strong indications that the attachment of the spermatozoon to the egg results in an action potential being propagated over the egg surface, the action potential being characterized by having no recovery phase." Scheer *et al.* (1954), although they could not obtain a steady membrane potential with an electrode apparently inserted into eggs of *Paracentrotus lividus* and *Arbacia lixula,* reported transient potential differences upon fertilization. These changes consisted of a series of rapid pulses of irregular size, ranging from about 2 to 5 mv. They began at the time that the first visible reaction (cortical change) to the sperm was observed, persisted during the period of egg contraction and gradually disappeared. In the case of *Paracentrotus* eggs the pulses were much less frequent (often only one or two) than in *Arbacia* (as many as fifty). Scheer *et al.* (1954) point out that these changes are not strictly comparable to the action potentials of nerve and muscle. Furshpan (1955) saw no potential changes upon fertilization of eggs of the sea urchins *Strongylocentrotus purpuratus* and *Lytechinus pictus,* but as in the case of the other observers, neither was a steady membrane potential obtained with these eggs. Similarly Kamada and Kinosita (1940), using an "internal" electrode found no change upon fertilization, nor a "resting" potential in eggs of the sea urchin *Strongylocentrotus pulcherrimus.* An attempt to examine possible changes in membrane potential upon fertilization was also made recently by Allen, Lundberg and Runnström (1955) with external electrodes in contact through sea water in agar with the ends of a capillary tube in which a sea urchin egg was elongated and fertilized. They found no shift in potential but concluded that the technique proved inadequate for this problem.

The present series of experiments disclose that in eggs of *Asterias forbesii,* at least, there is a change in the membrane potential beginning with a sudden decrease when spermatozoa are seen to have made contact with the egg. The initial decrease of membrane potential is converted to an increase which reaches its maximum and steady value when the fertilization membrane is raised. These changes in membrane potential appear to develop smoothly without the occurrence of the pulses recorded by Scheer *et al.* (1954) with a capacitatively coupled amplifier.

The time course of the initial decrease in potential was not determined accurately. The optical system did not permit identification of the moment of sperm entry into the egg, and unfortunately photographic recording of the oscillograph traces was not done at sufficiently frequent intervals to define accurately the initial portion of the membrane potential change. However, visual observation at 1 per second sweeps, which was routinely done, indicated that the decrease took less than 10 seconds to reach its maximum value.

More detailed experiments will be required to establish whether or not the observed electrical changes in *Asterias* eggs are associated in time with the optical changes which accompany fertilization. The color change which passes over the surface of the sea urchin egg within a few seconds after the attachment of the sperm (Runnström, 1928) is a propagated response with a total conduction time of about 20 seconds at 18° C. (Rothschild and Swann, 1949). Birefringence of the surface

disappears in about the same time (Monroy and Montalenti, 1947). These events therefore have about the same time course as the decrease and subsequent beginning of the return of the membrane potential to its former steady amplitude.

Studies on the rate at which block to polyspermy is established in sea urchin eggs (Rothschild and Swann, 1950, 1951, 1952; Rothschild, 1953, 1954) have led to the conclusion that there is a fast reaction which passes over the surface of the egg in about two seconds and that reduces the chances of refertilization by a factor of 20. This is followed by a slower change which in about 60 seconds reduces to zero the probability of a successful sperm-egg collision. The wave of explosion of the "Harvey-Moser granules" (Harvey, 1911, p. 523; Moser, 1939) takes about 15 seconds to traverse the surface of an echinoid egg from the point of attachment of the sperm (Endo, 1952). The time course of these events is about the same in the starfish egg judging from the fact that the fertilization membrane becomes visibly elevated at one to two minutes. As noted earlier, the new maximum of membrane potential is attained at about the time that the fertilization membrane is clearly visible.

A distinction must, however, be made in correlating the electrically and optically observed effects. The latter are initiated at the site of entry of the sperm and are slowly propagated from there. The former are recorded with an electrode inside a sphere of small diameter and this condition operates against the likelihood of observing discretely localized membrane potential change. If the electrical changes observed during fertilization have their basis in localized changes of the membrane, these are probably electronically averaged in the actual recording and would not reveal clearly a spreading electrical change which might accompany the propagated evolution of the optically observed phenomena.

The nature of the membrane events which lead to the electrical changes observed after fertilization can only be speculated upon. Potential change in excitable tissues is associated with change in membrane permeability and/or altered ionic flux (Bernstein, 1912; Hodgkin and Katz, 1949; Fatt and Katz, 1951; Hodgkin and Huxley, 1952; Eccles, Fatt and Koketsu, 1954). In the more complex bioelectric generators of frog skin or the gastric mucosa, changes in the transport respectively of Na^+ (Ussing, 1954; Kirschner, 1955) and Cl^- (Hogben, 1955) are involved. At least in the former case, metabolic activity and hormonal factors play an important role. Whether or not the electrical changes which occur upon fertilization are associated with known metabolic changes (*cf.* Brachet, 1947; Runnström, 1949; Tyler, 1955a) is not at present clear. Changes in ionic permeability such as might be indicated by the electrical data probably do occur. However, the time course of the K^+ accumulation is not known. Monroy-Oddo and Esposito (1951) have reported that sea urchin eggs gain K^+ upon fertilization, and such accumulation might account for the late increase in the membrane potential. Malm and Wachmeister (1950), on the other hand, report a slight decrease in potassium content, and a considerable increase in sodium, of sea urchin eggs upon fertilization. They attribute this to the fertilization membrane being permeable to the ions in the surrounding sea water, so that analyses after fertilization would show an increase in ions present in high concentration in sea water and an apparent decrease in ions like potassium previously accumulated by the egg. Shapiro and Davson (1941) had also reported no significant change in potassium content in sea urchin eggs upon fertilization. They noted, too, that both unfertilized and fertilized eggs lost potassium slowly

(1.5 to 8% in two hours) on standing in sea water, and that eggs in K^+-enriched (5 ×) artificial sea water accumulated K^+. In experiments with radioactive cations Brooks (1939) noted an increase in the accumulation of radiosodium upon fertilization in eggs of *Urechis caupo*. E. L. Chambers *et al.* (1948) reported that the rate of exchange of K^{42} increased 7 to 13 times upon fertilization in eggs of *Strongylocentrotus purpuratus* and *Arbacia punctulata*. They considered (see also E. L. Chambers, 1949) that only 20 per cent of the K is readily exchangeable in the unfertilized egg and 85 to 100 per cent in the fertilized egg. The rate of exchange of "freely diffusible" K is therefore concluded to be two to three times more rapid in the fertilized egg. While there are some evident differences in the results of the various experiments cited, the indications are that there are likely to be changes in permeability to certain ions, occurring upon fertilization, that may correlate with the changes in electrical potential.

On the other hand, the electrical changes might rather be due to alteration in the mechanical properties of the egg membrane. The large potential initially seen on penetrating the unfertilized egg was about equal in magnitude to the largest steady potential attained after fertilization. The decline from the initial value has been interpreted as being caused by imperfect sealing of the plasma membrane around the shaftlet of the microelectrode. It is therefore possible that the initial decrease upon fertilization indicates a further loosening of the seal and that the subsequent rise of membrane potential only reflects formation of a better seal. The various physical changes initiated in the fertilization reaction and discussed above might well be implicated in an alteration of the membrane seal around the electrode.

The electrical constants of the egg membrane

The measurements of membrane resistance and capacity were in the present experiments subsidiary to the use of the square pulse technique for ascertaining penetration of the egg surface by the microelectrodes. Therefore the results are chiefly indicative of the orders of magnitude of these values, subject to a more extensive study. However, the measurements can throw some light on the more detailed interpretation of the membrane potential and will be discussed largely in this context.

The unfertilized egg

Most cells thus far adequately studied have membrane resistances in the range of 1000 ohm-cm.^{2} However, the values range from as low as 0.1 ohm-cm.^{2} (rostral membrane of the electoplaque of the eel; Keynes and Martins-Ferreira, 1953) to as high as 20,000 ohm-cm.^{2} (activated *Fundulus* egg; Kao, 1955). The membrane resistance of *Asterias* eggs (average: 3,100 ohm-cm.^{2}) indicates that ionic transport is rather low across this membrane. In a tabulation of the membrane resistance of various cells, Cole and Curtis (1950) give an estimate that the membranes of both sea urchin and starfish eggs have infinite resistance. Earlier, however, Cole and Cole (1936a) pointed out that the method of measurement was not appropriate for determinations of membrane resistance. A 2% change in the estimate of relative cell volumes to that of external fluid would have led to a calculated value of membrane resistance as low as 25 ohm-cm.^{2} Rothschild (1938) stated that while his attempts to measure membrane resistance of *Echinus* eggs were unsatisfactory, the

value was probably no higher and perhaps lower than 10^4 ohm-cm.^{2} Furshpan (1955) estimated that the resistance which the sea urchin egg interposed in the microelectrode circuit of his experiments was about 1 megohm. From the surface area of these eggs (diameter, 75 μ; area 1.8×10^{-4} cm.^{2}) he calculated the membrane resistance as 180 ohm-cm.^{2} However, since no membrane potential was obtained in those experiments, it is unlikely that the electrode had penetrated the egg membrane and this might account for the low estimate. Cole and Curtis (1938) calculated membrane resistances from 0.2 to 10 ohm-cm.^{2} for unfertilized and fertilized *Arbacia* eggs from measurements of single eggs in a small capillary but were forced to discard them because the assumption of no parallel leakage also gave unreasonable values for the membrane capacity. Allen, Lundberg and Runnström (1955) report a resistance of 8.7×10^5 ohms for an egg of *Psammechinus* in a narrow (57 μ) capillary but consider that this may be in error by virtue of a leakage pathway provided between the surface of the egg and the walls of the capillary. They state (p. 178) that in later experiments Lundberg (1955, unpub.) has obtained a value of 1350 ohm-cm.^{2}

Passage of currents of 0.5 to 1.5 μA through the *Asterias* egg, corresponding to current densities of 0.75 to 2.25 mA/cm.^{2}, caused marked decrease of the membrane resistance in 6 eggs. These results need further study, and if the phenomenon is established might yield valuable clues to membrane properties, since the change in resistance was, at least in some eggs, not accompanied by cytolysis. The relatively short pulses used in the experiments did not appear to cause any activation changes such as were reported in the experiments of Allen, Lundberg and Runnström (1955).

It is interesting to note that even with the highest currents employed, the relation between membrane current and voltage was strictly linear both for inward and outward currents. This is not the case in excitable tissues. Outwardly directed currents of relatively low magnitude initiate the changes of membrane potential inherent in the local response and spike and these are associated with a marked drop of membrane resistance, whereas inwardly directed currents tend to increase the resistance (Cole and Curtis, 1941). Thus, eggs of *Asterias*, although they undoubtedly undergo excitation by sperm in the form of the reactions of fertilization, evidently do not respond to electric stimulation in the manner characteristic of electrogenic excitable tissues.

It is of further interest that the maximum quantity of electricity in the square pulses used for the present experiments was about 5×10^{-5} coulomb/cm.^{2} The squid giant axon and other cells, however, are excited by 10^{-8} to 10^{-9} coulomb/cm.^{2} of membrane (Cole, 1949; Grundfest, 1952; Hodgkin, Huxley and Katz, 1952).

Effect of fertilization

If the initial decrease and subsequent increase of the membrane potential in the fertilized egg are consequences of altered ionic flux, the latter change should be reflected as a change of the membrane resistance. The most accurate measurements of this with respect to both magnitude and time course would be provided by study of the high frequency impedance. Such measurements were not done in the present experiments and the values derived from the square pulse technique apply, not to the initial period of fertilization, but to the stage when the fertilization membrane

had been lifted and the membrane potential had reached its steady higher value. At this time the membrane resistance of the two eggs studied before and after fertilization (Fig. 8, Table 4A) was identical with the initial value. This may be taken to indicate that the fertilization membrane is a rather porous structure which offers relatively little impedance to ion movements. Cole (1928), Cole and Spencer (1938) and Cole and Guttman (1942) reported that upon fertilization of sea urchin or frog eggs there was no change in cortical resistance, and our data therefore also support this finding as regards at least a stage a few minutes after fertilization.

However, this constancy poses a dilemma which we have not been able to resolve. It was suggested earlier that the low value of steady membrane potential in the unfertilized egg, and its initial decrease and subsequent increase upon fertilization, might have been caused by alterations in the seal of the egg membrane against the wall of the microelectrode. This assumption would necessitate upward revision of the value of the membrane resistance of the unfertilized egg, perhaps up to double the calculated average of 3100 ohm-cm.^{2}, since the leaks decreasing the membrane potential to about half would be in parallel with the resistance of the membrane. However, the membrane resistance of the eggs seemed to be relatively independent of their steady membrane potentials (Table IVA), and therefore no attempt has been made in the present study to correct the calculated values of R_M. Nevertheless, the possibility remains that the membrane resistance of the unfertilized egg is higher than 3000 ohm-cm.^{2} and that the apparent absence of change after fertilization is fortuitous. The improved sealing of the membrane to the electrode might have increased the leakage resistance greatly and thereby have improved the accuracy of the measurement of R_M. In that case, the actual membrane resistance of the egg may have decreased upon fertilization. More extensive studies will be required to resolve this matter. However, the data, whether the resistance of the membrane is constant or decreases, differ from the finding that the membrane resistance of activated *Fundulus* eggs increases 4- to 7-fold (Kao, 1955). The difference may be explained by the fact that in *Asterias* the egg volume and surface remain nearly constant after fertilization, whereas the activated *Fundulus* egg shrinks markedly. The resultant diminution of surface may therefore lead to closer packing of the ion-permeable units of the plasma membrane (Kao, Chambers and Chambers, 1954), or the effective narrowing of pores in the membrane.

Cole (1938) reported a 100% rise in cortical capacitance of fertilized *Arbacia* and *Hipponöe* eggs and Cole and Spencer (1938) report 300% in *Arbacia*. In the two *Asterias* eggs studied before and after fertilization, the calculated membrane capacity after fertilization remained unaltered in one and decreased in the other. As noted earlier, the accuracy of the measurements of the time constant was not as high as is either desirable or attainable and this matter must be left open subject to future work.

Summary

1. The paper describes electrical characteristics of the egg of the starfish *Asterias forbesii* as measured with a microelectrode penetrating the surface. The study included the effects of fertilizing the egg while the latter was impaled on the electrode.

2. It has been confirmed that penetration of the egg membrane cannot be indicated solely by the seeming visualization of the microelectrode at the center of the egg.

3. A method involving use of two microelectrodes is described for ascertaining penetration of the egg surface. One internal electrode delivers a current pulse and the other records the time course of the resultant membrane IR drop.

4. Contrary to the reports of many earlier investigations on echinoderm and other eggs a potential difference is found upon penetration of the unfertilized egg.

5. The potential difference at the time of penetration amounts to about 60 mv., inside negative, but this soon decreases to lower steady values ranging from − 10 to − 50 mv. in different eggs.

6. Upon insemination of the impaled egg the membrane potential abruptly decreases by 5 to 10 mv. when sperm are seen to have reached the egg, then rises during the ensuing 1 to 2½ minutes, as the fertilization membrane is raised, reaching a new steady value 5 to 20 mv. greater than that of the unfertilized egg.

7. The possible basis of these changes is discussed.

8. The internal K^+ of unfertilized *Asterias* eggs is from 17 to 21 times higher than that of sea water. The sodium determinations, while subject to larger error, indicate a concentration less than 5% that of sea water.

9. The membrane potential of either unfertilized or fertilized eggs decreases when the external K^+ is raised and returns to the original value when the excess K^+ is removed.

10. As in many other kinds of cells the potential is evidently a consequence primarily of the high internal concentration of, and permeability to, K^+, but the magnitude appears less than predicted by the Nernst equation.

11. The membrane resistance of the unfertilized egg averages 3100 ohm-cm.^{2}, but might be higher on the assumption of possible leaks around the microelectrode.

12. The measured resistance is unchanged after fertilization, but, on the assumption of the formation of a tighter electrical seal the actual membrane resistance would be lower.

13. The membrane capacity is of the order of 0.5 μF/cm.^{2}

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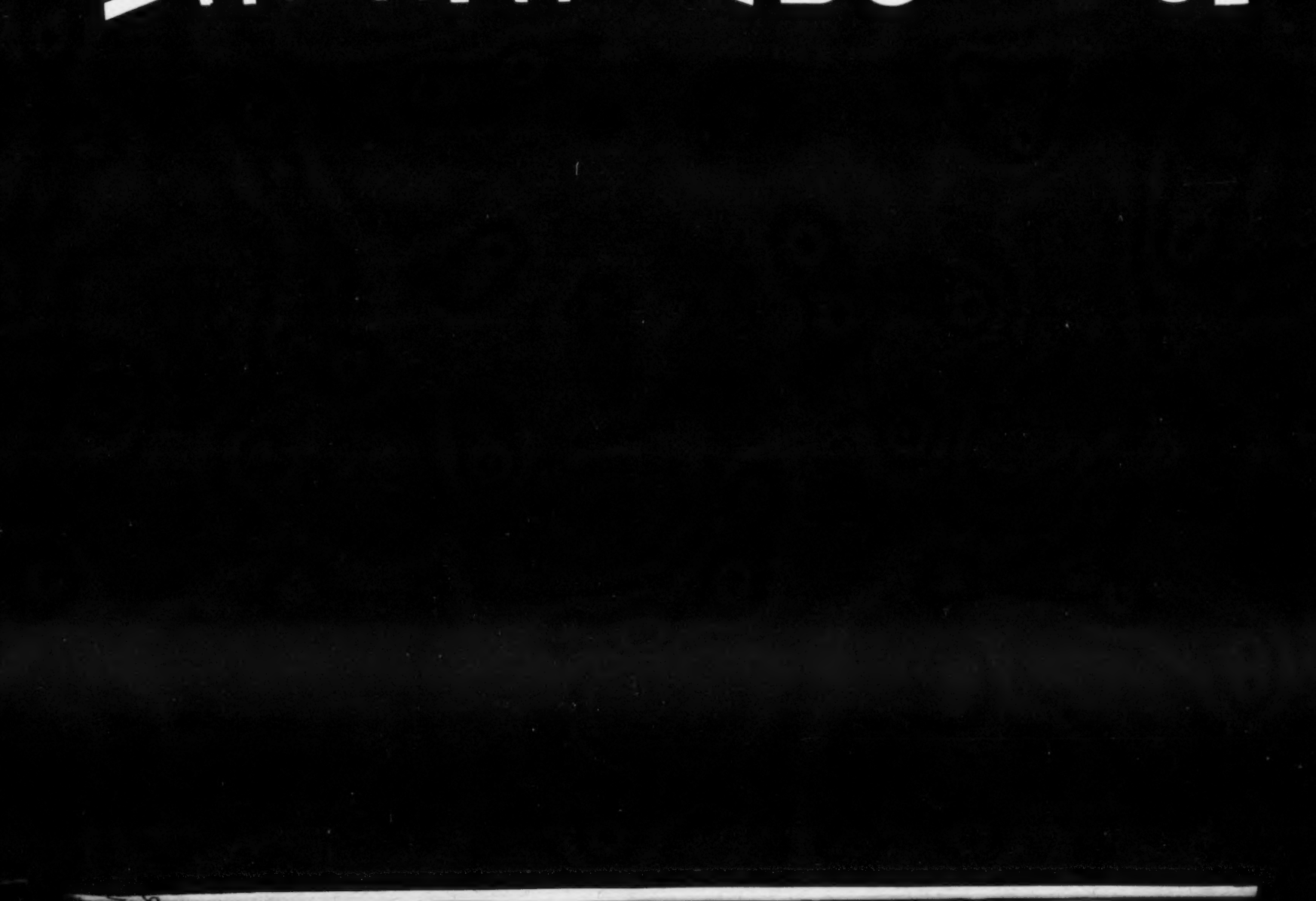
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